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Caffeine, Adenosine Receptors and Estrogen in Toxin Models of Parkinson's Disease

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**14. ABSTRACT**

Substantial progress has been made toward our original Specific Aims (SAs) in pursuit of the core hypothesis that *multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in PD*. SA#1: Using a series of global and conditional knockout mice lacking the adenosine $A_{2A}$ receptor, we characterized the molecular and cellular basis of caffeine's protective actions in acute toxin models of PD. Recently we showed that caffeine also confers protection in a chronic pesticide model of PD. SA#2: We have validated a powerful (AAV) virus-based (*cre*) gene delivery system to conditionally knock out adenosine receptors from specific brain regions, enabling us to determine in which brain region(s) $A_{2A}$ receptors contribute to neurotoxicity. Despite technical challenges of viral and/or *cre* gene toxicity, we have obtained initial evidence of striatal $A_{2A}$ receptor-dependence of MPTP toxicity with this system. SA#3: We systematically demonstrated that endogenous estrogen (in females) and exogenous estrogen (e.g., in ovariectomized females) can prevent the protective effect of caffeine on MPTP toxicity, without altering caffeine pharmacokinetics. The findings provide insight into epidemiological studies consistently identifying coffee and caffeine consumption as an inverse risk factor for PD in men, and only in women who have not taken estrogen replacement therapy. These data have helped substantiate that critical interactions between environmental toxicants and protectants influencing the neurodegeneration of PD. Finally, our demonstrations of adenosine $A_{2A}$ receptor-dependent protection by caffeine, and of the prognostic biomarker potential of another purine urate, have directly contributed to the rationale for novel clinical trials with disease-modification designs currently in progress.

**15. SUBJECT TERMS**
adenosine, caffeine, estrogen, neuroprotection, neurotoxin, Parkinson's disease

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# Introduction

Identifying the mechanisms by which purines like caffeine and more specific $A_{2A}$ antagonists protect dopaminergic neurons in models of Parkinson's disease (PD) will advance our knowledge of the pathophysiology, epidemiology and therapeutics of PD.

The *overarching hypothesis* pursued by this proposal is that **multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease**. Here we endeavor to characterize the interplay between several environmental agents (pesticides, caffeine, estrogen and most recently the caffeine-related purine urate) that are leading candidate modulators of PD risk.
We have pursued 3 *specific hypotheses:*

**1)** Caffeine acts through blockade of brain $A_{2A}$Rs to protect dopaminergic neurons in both acute and chronic toxin models of PD. (***Specific Aim #1***)

**2)** Caffeine perfusion and focal $A_{2A}$R inactivation within the striatum are sufficient to attenuate MPTP toxicity, by reducing toxin-induced release of glutamate and/or GABA. (***Specific Aim #2***)

**3)** Estrogen attenuates the protective effect of caffeine but not the protective of $A_{2A}$R deletion because it acts by altering caffeine metabolism or $A_{2A}$R expression. (***Specific Aim #3***)

## Statement of Relevance (from original proposal)

### A. Parkinson's Disease -

**Basic neuroscience significance** - The results will improve our understanding of adenosine receptor neurobiology, and will provide insight into the role of endogenous adenosine in basal ganglia biology physiology and PD pathophysiology.

**Epidemiological significance** - Establishing the ability of caffeine to protect dopaminergic neurons in PD models and identifying a plausible mechanism of action greatly strengthens the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with risk of PD.

**Therapeutic significance** - With several specific adenosine $A_{2A}$ antagonists emerging as promising therapeutic candidates based on their motor-enhancing (symptom-relieving) action, the prospects for additional neuroprotective benefit substantiated by this project may considerably enhance their therapeutic potential. In addition, identifying a biological basis for caffeine-estrogen interaction in modifying PD risk could also affect recommendations for estrogen replacement strategies in women with PD taking $A_{2A}$ antagonists or caffeine (and *vice versa*). Furthermore, based on evidence that $A_{2A}$Rs contribute to the neurotoxicity affecting cortical and striatal neurons (as well as dopaminergic neurons), our findings may support novel $A_{2A}$R-

based neuroprotective treatments for a wider range of neurological diseases from stroke to amyotrophic lateral sclerosis (ALS) to Alzheimer's disease.

**B. Environmental Neurotoxin Exposure in Military Service** – By characterizing the neuroprotective effects of caffeine in a chronic pesticide model of PD (as well as the acute MPTP model), the proposed work will define a prototypical interaction between environmental toxins and protectants in determining the extent of a well-characterized neurological lesion (dopaminergic neuron death). Although there has been no compelling evidence to suggest that the incidence of PD will itself increase in association with military service or combat theatre exposures,[1] putative toxin exposure in the military may be linked to the development of another debilitating neurodegenerative disorder, ALS.[2] Moreover, some objective biological measures in veterans diagnosed with a "Persian Gulf War syndrome" have indicated dysfunction of dopaminergic neurotransmission in the basal ganglia,[3] raising the possibility (together with other data[4]) of altered risk for PD in this group. In any event, establishing a biological precedent for neurotoxin-neuroprotectant interplay in the relatively common disorder of PD, may provide a 'roadmap' that can be used should any neurological illness be confirmed to develop in association with prior military exposures.

**C. Understanding the Non-stimulant CNS Effects of Caffeine.** The psychoactive agent caffeine has been endorsed for military use at relatively high doses to help maintain operational readiness.[5] This recommendation has been based on a large body of evidence demonstrating sustainment of mental task performance by caffeine, and a lack of evidence for substantial harm at these doses. However, adopting the use of any CNS-active drug by protocol warrants careful consideration of newly appreciated neuronal actions of the agent. Accordingly, the proposed investigation of the novel neuroprotective effect of caffeine and its underlying mechanisms (e.g., altered neurotransmitter release) would be of significance for military programs that provide specific doses of caffeine to personnel to enhance cognitive function.

**D. Gender Differences in How Environmental Factors Impact Toxin Susceptibility.** Our investigation of how caffeine and estrogen exposures interact to modify neurotoxin susceptibility in laboratory models of PD may have substantial significance for the human epidemiology that prompted our pursuit of this line of research. In addition, the proposed studies may provide a prototype for modeling how gender and estrogen status interact with environmental exposures of relevance to the military (i.e., neurotoxins, caffeine). A better appreciation of how gender alters susceptibility to environmental toxins or protectants may ultimately lead to a better understanding (and modification) of the differential risks faced by women and men serving in the same military operations.

# Body of the Report: from Yr 1 Annual Report

Progress on Specific Aims and experiments as laid out in our Statement of Work (SOW [in blue]) is described here in detail.

## STATEMENT OF WORK

***Specific Aim #1*** – to definitively determine whether brain $A_{2A}$Rs or $A_1$Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain $A_{2A}$R is required for caffeine's protective effect in these PD models. **(~576 mice)**

*Hypothesis 1*: **Caffeine acts through blockade of brain $A_{2A}$ (not $A_1$) receptors to protect dopaminergic neurons in both acute (MPTP) and chronic (paraquat/maneb) toxin models of PD.**

*Exp# 1* – **Effect of the $A_1/A_{2A}$ receptor double KO in MPTP and paraquat/maneb (Pq/Mb) models**

We have generated an $A_1$-$A_{2A}$ double KO line of mice in collaboration with Drs. Jiang-Fan Chen (Boston) and Bertil Fredholm (Sweden) in which our previously characterized $A_{2A}$ KO mice have been crossed with $A_1$ KO mice. Importantly, we designed a breeding scheme that has placed the individual KO lines onto a common genetic background (C57Bl/6) using a marker-assisted selection ("speed congenic") strategy that virtually eliminates the common confounder of mixed genetic background in the interpretation of knockout mouse studies. During the past year we have begun to expand the colony through double heterozygote ([$A_1^{+/-}$, $A_{2A}^{+/-}$] x [$A_1^{+/-}$, $A_{2A}^{+/-}$]) matings. This breeding strategy though arduous yields well-matched set homozygous $A_1$ KO [$A_1^{-/-}$, $A_{2A}^{+/+}$], $A_{2A}$ KO [$A_1^{+/+}$, $A_{2A}^{-/-}$], double $A_1$-$A_{2A}$ KO [$A_1^{-/-}$, $A_{2A}^{-/-}$] and control WT [$A_1^{+/+}$, $A_{2A}^{+/+}$] littermates. (Note that mice of these 4 genotypes comprise only a quarter of all offspring.)

In preparation for the proposed experiments we are expanding this double KO colony through these double heterozygote crosses, with PCR genotyping of the offspring producing the desired KOs and controls in the expected Mendelian distribution as shown in Figure 1 (with lane 2 showing a double KO, for example). We are also completing a stereological analysis of nigral dopaminergic neurons after chronic treatment *in vivo* with the paraquat/maneb pesticide combination (+/- caffeine, a mixed $A_1$-$A_{2A}$ antagonist), which may further establish the rationale for investigating this chronic pesticide model of PD in the single and double $A_1$-$A_{2A}$ KO mice.

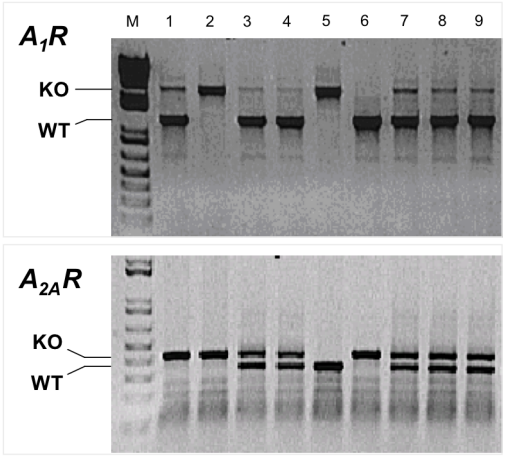



Fig. 1 – ***$A_1R$* and *$A_{2A}R$* genotyping of offspring from double heterozygote matings.** To detect WT and mutant (KO) alleles of the *$A_1R$* and *$A_{2A}R$* genes in the same congenic C57BL/6mice, we employed PCR genotyping protocols with one downstream primer targeting the inserted *Neo*-cassette and another targeting the WT *$A_1R$* or *$A_{2A}R$* gene to distinguish mutant from WT alleles for each gene.

### *Exp# 2* – Effect of brain-specific $A_{2A}$ KO in MPTP and Pq/Mb models.

In preparation for this experiment we have completed the generation and initial characterization of a conditional (Cre/*loxP* system) KO of post-natal forebrain neuronal $A_{2A}$ receptors. As detailed in our proposal the *CamKII*$\alpha$ promoter was used to drive expression of the *cre* recombinase gene in postnatal forebrain neurons, and thus to cause selective depletion of striatal neuron $A_{2A}$ receptors following brain development. In our recent publication Bastia et al, 2005 (*Neuropsychopharm*, 30:891-900) we confirm successful forebrain-specific recombination by genetic, autoradiographic and behavioral assessments.

### *Exp# 3* – Brain $A_{2A}$R-dependence of caffeine's neuroprotective effect.

We took advantage of a complementary transgenic Cre mouse line (provided by Dr. David Guttmann, St. Louis) that uses the *GFAP* promoter to drive expression of the *cre* gene in astrocytes. By crossing this mouse with the 'floxed $A_{2A}$' line of mice, we have generated a set of mice lacking the $A_{2A}$ receptor in astrocytes. This astrocytic $A_{2A}$ KO line has allowed us to ask whether brain astrocytes express the $A_{2A}$ receptors whose blockade by caffeine protects against MPTP toxicity. This possibility has become of particular interest with the recent appreciation that astrocyte $A_{2A}$ receptors can play a role in control extracellular glutamate concentrations.[7-8]

This past year we have conducted two experiments to address the role of astrocyte $A_{2A}$ receptors in neuroprotection by caffeine. The pooled results (see Fig. 2) clearly indicate that the astrocyte $A_{2A}$ receptor is not essential for caffeine's protective effect in the MPTP model because it's neuroprotection by caffeine is undiminished in mice lacking astrocyte $A_{2A}$ receptors. This finding is consistent with our original hypothesis, and we therefore plan to pursue the neuron-specific conditional KO experiment in the coming year.

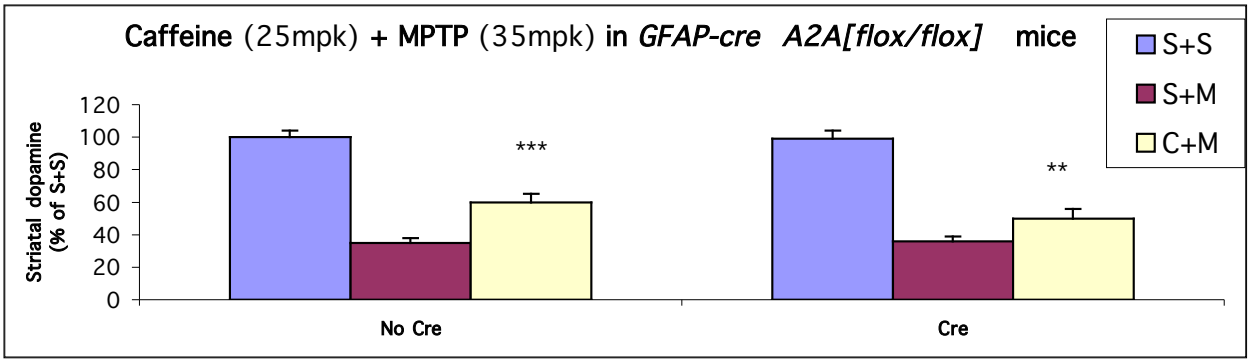



Fig. 2 – **Conditional astrocyte KO of $A_{2A}$ receptors does not eliminate neuroprotection by caffeine.** Caffeine (**C**) attenuates the loss of striatal dopamine induced by MPTP (**M**, 35 mg/kg) not only in control ("**No Cre**") mice (i.e., *$A_{2A}$[flox/flox]*), but also in astrocyte (conditional) KO littermates ("**Cre**") mice (i.e., *GFAP-cre*, *$A_{2A}$[flox/flox]*).

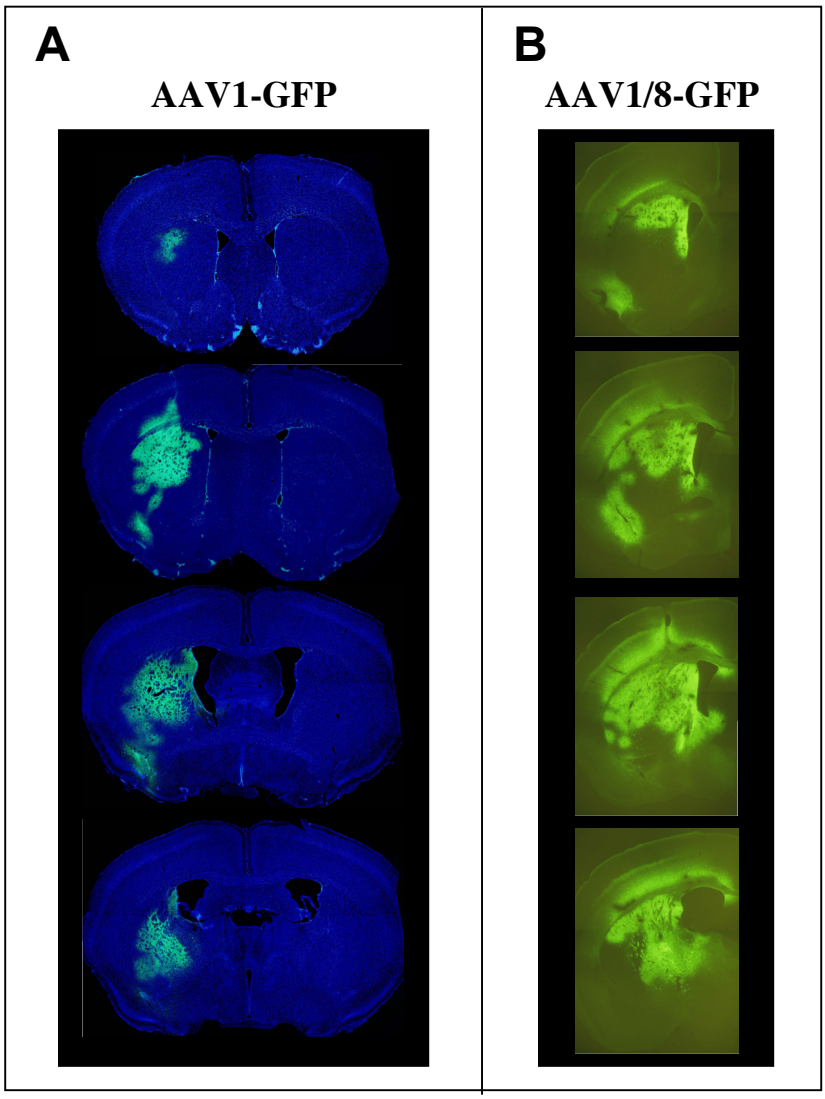
***Specific Aim #2*** – to localize the region within brain through which caffeine or $A_{2A}$ receptor inactivation produces its neuroprotective effect in the MPTP model of PD. **(~576 mice)**

*Hypothesis 2:* **Caffeine perfusion and focal $A_{2A}$ receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

*Exp# 4* – **Effect of intracerebral caffeine perfusion on MPTP-induced neurotransmitter overflow and toxicity**:

In preparation for this experiment with local administration of caffeine, we are continuing to characterize (using microdialysis) MPTP-induced neurotransmitter overflow in the striatum and its modulation by systemic caffeine.

*Exp# 5* – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed $A_{2A}$R mice**: Homozygous floxed $A_{2A}$R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP. One week later infusion needle track will be localized histochemically, while dopaminergic neuron integrity will be visualized by striatal DAT binding and nigral TH-IR counts will be assessed as in Exp #4.




We have made substantial progress toward this experiment in successfully introducing the a viral (AAV) *cre* recombinase gene into the striatum of 'floxed $A_{2A}$' mice, leading to the unilateral local

recombination and disruption of the $A_{2A}$ gene, and in turn the elimination of striatal $A_{2A}$ receptors. Working with several serotypes of AAV-*cre* and AAV-*GFP* (provided through a collaboration with Dr. Miguel Esteves), Dr. Augusta Pisanu (a post-doctoral fellow who joined the lab earlier this year specifically to pursue this SA) has demonstrated that AAV-*GFP* stereotactically injected into the striatum of wild-type mice leads to the local expression of GFP (green fluorescent protein; Fig. 3). The area of infection/expression was better localized to the targeted striatum when using a serotype 1 AAV-*GFP* (Fig. 3A) compared to a mixed serotype 1/8 AAV-*GFP* (which produced more widespread expression crossing over from the targeted striatum into the overlying cortex; Fig. 3B).

Fig. 3 – **Intrastriatal injection of AAV1-*GFP* leads to local striatal GFP expression.** (**A**) One month after stereotactic unilateral intrastriatal injection of AAV1-*GFP* into wild-type C57BL/6, coronal brain sections were treated with a Hoechst chromatin stain (rich blue background). Green fluorescence was detected in rostral (top) through caudal (bottom) sections only on the side of the injection, and almost entirely within the striatum (**str**). (**B**) By contrast, injection of an AAV1/8-GFP serotype under the same conditions led to a much less discreet distribution of GFP (without Hoechst counter-stain in this mouse) in cortex (**ctx**) as well as striatum. Injection needle tracks are indicated (*).

Accordingly, we have selected AAV1-*cre* (serotype 1 AAV-*cre*) for targeting the selective elimination of striatal $A_{2A}$ receptors in floxed $A_{2A}$ mice. Figure 4A shows successful *cre* expression (with detection of Cre immunoreactivity within striatal nuclei by immunohistochemistry) on the side of AAV1-*cre* injection. Remarkably, adjacent coronal sections demonstrate complete elimination of detectable striatal $A_{2A}$ receptors (Fig. 4B) in the exact region of the Cre recombinase. This close correlation of nuclear Cre expression and $A_{2A}$ receptor disappearance is consistent with functional recombinase producing the expected recombination across the *loxP* sites flanking a critical sequence within the $A_{2A}$ gene in the 'floxed $A_{2A}$' mice. This sequence deletion was designed to disrupt the gene, leading to discontinued production of functional receptor.

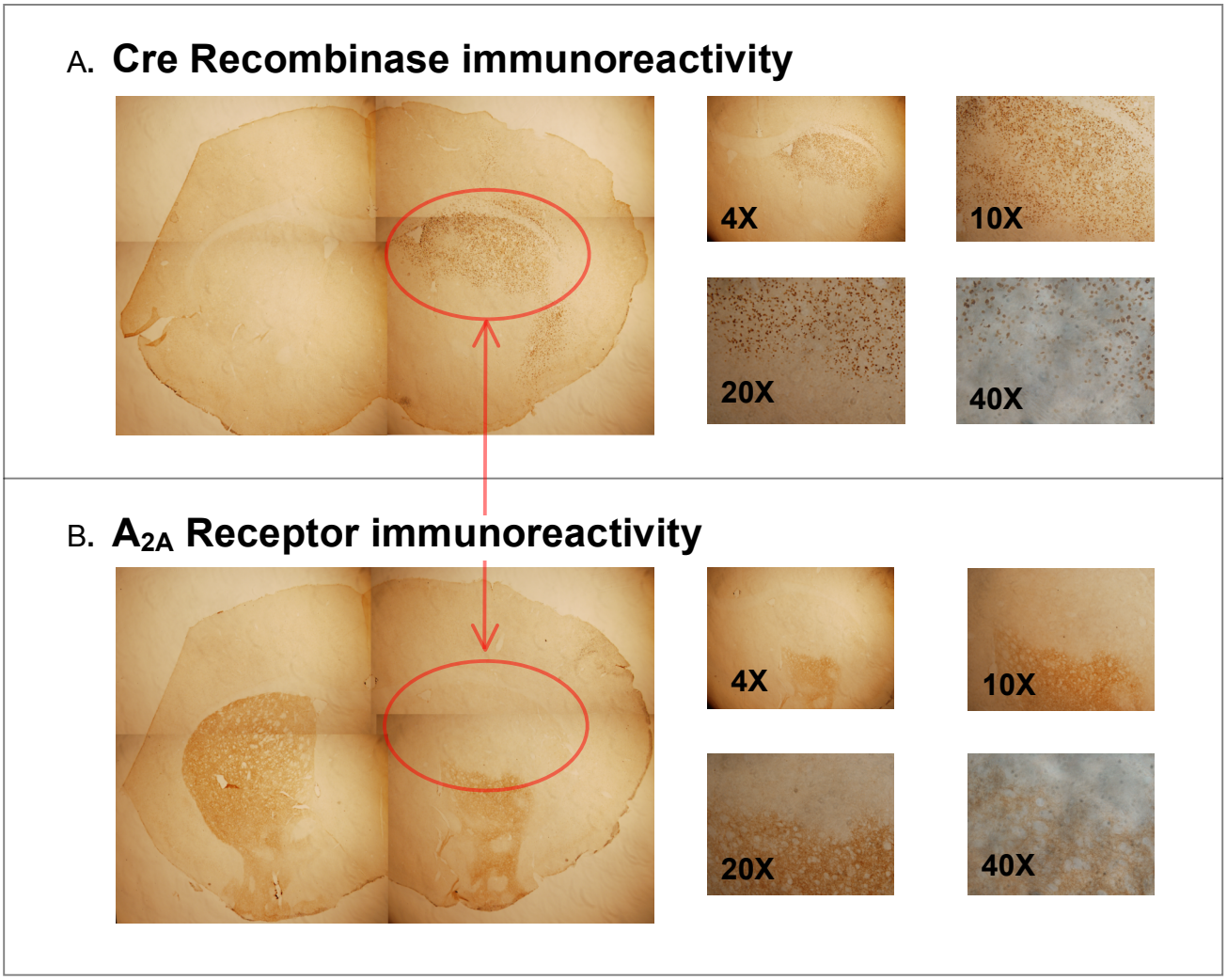



Fig. 4 – **Intrastriatal injection of AAV1-*cre* leads to local striatal Cre expression (A) and the coincident elimination of striatal $A_{2A}$ receptors (B) in 'floxed $A_{2A}$' mice.** One month after stereotactic unilateral intrastriatal injection of AAV1-*cre* into 'floxed $A_{2A}$' (*$A_{2A}$[flox/flox]*) mice, Cre-immunoreactivity was assessed by Cre immunohistochemsitry (**A**) with a HRP-coupled secondary antibody yielding a (brown) reaction product in the nucelei of striatal cells (seen best at 40x magnification on the right). Adjacent coronal brain sections were processed for $A_{2A}$ receptor immunohistochemsitry (**B**) with a HRP-coupled secondary antibody yielding a (brown) DAB reaction product more but specifically staining the striatum, except in the precise location of Cre expression (c.f., contents of red ovals in **A** and **B**).

This methodological advance will allow us to dissect $A_{2A}$ receptor involvement in neurotoxin models of PD with an unprecedented combination of anatomical and molecular precision.

***Specific Aim #3*** – to investigate caffeine-estrogen interactions in the MPTP model of PD by determining the effect estrogen replacement on the neuroprotective phenotype of $A_{2A}$ KO mice, and exploring potential peripheral and CNS mechanisms contributing to caffeine's reduced neuroprotective efficacy in the presence of estrogen. **(~576 mice)**

With support from this award we have completed our initial study of estrogen-caffeine interaction in the MPTP model of Parkinson's disease, as detailed in the attached manuscript and meeting abstract (Appendices A and N). Our results demonstrate that estrogen reduces caffeine's neuroprotective effect against MPTP toxicity in both male and female mice. In the context of human epidemiology on PD, our findings suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

*Hypothesis 3:* **Estrogen attenuates the protective effect of caffeine but not the protective of $A_{2A}$ receptor deletion because it acts by altering caffeine metabolism or $A_{2A}$ receptor expression.**

*Exp# 6* – **Effect of estrogen replacement on MPTP toxicity in OVX $A_{2A}$ KO versus WT mice**.

The clear outcome of *Exp# 7* showing that estrogen did not alter caffeine metabolism (see below) argues against the premise in *Hypothesis 3*. Therefore, it may be that estrogen will in fact attenuate the protective effect of $A_{2A}$ receptor depletion (in the $A_{2A}$ KO) as well as caffeine's protective effect.

*Exp# 7* – **Effect of estrogen replacement on caffeine pharmacokinetics and striatal $A_{2A}$ receptor density**: The effects of controlled-release estrogen versus vehicle pellets implanted in OVX female WT mice will be determined on serum and brain concentrations of caffeine at various time points after intraperitoneal caffeine administration (Exp # 7a), and on $A_{2A}$, $D_1$ and $D_2$ receptor autoradiography in the striatum (Exp # 7b).

We have completed this experiment with the clear demonstration that chronic exogenous estrogen attenuates caffeine's neuroprotective effect without altering serum or brain levels and kinetics of caffeine or its demethylation metabolites theophylline, paraxanthine and theobromine. These data are provided in the context of a full manuscript under revision for the *J. Neurosci*. (Appendices N and W). We similarly report that $A_{2A}$ receptor expression is unaffected by estrogen treatment in our paradigm.

# Body of the Report: from Yr 2 Annual Report

Progress during Year 2 on Specific Aims and experiments as laid out in our Statement of Work (SOW [in blue]) is described here in detail.

## STATEMENT OF WORK

***Specific Aim #1*** – to definitively determine whether brain $A_{2A}$Rs or $A_1$Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain $A_{2A}$R is required for caffeine's protective effect in these PD models.

**[Please see abstract publications in Appendices D and E.]**

*Hypothesis 1:* **Caffeine acts through blockade of brain $A_{2A}$ (not $A_1$) receptors to protect dopaminergic neurons in both acute (MPTP) and chronic (paraquat/maneb) toxin models of PD.**

*Exp# 1* – **Effect of the $A_1/A_{2A}$ receptor double KO in MPTP and paraquat/maneb (Pq/Mb) models**

As reported in our Year 1 progress report we have generated an $A_1$-$A_{2A}$ double KO line of mice in collaboration with Drs. Jiang-Fan Chen (Boston) and Bertil Fredholm (Sweden). The expansion of this line through double heterozygote ([$A_1^{+/-}$, $A_{2A}^{+/-}$] x [$A_1^{+/-}$, $A_{2A}^{+/-}$]) matings has been slow yielding adequate numbers of well-matched set homozygous $A_1$ KO [$A_1^{-/-}$, $A_{2A}^{+/+}$], $A_{2A}$ KO [$A_1^{+/+}$, $A_{2A}^{-/-}$], double $A_1$-$A_{2A}$ KO [$A_1^{-/-}$, $A_{2A}^{-/-}$] and control WT [$A_1^{+/+}$, $A_{2A}^{+/+}$] littermates. (Note that mice of these 4 genotypes comprise only a quarter of all offspring.) The generation of $A_1$ KO [$A_1^{-/-}$, $A_{2A}^{+/+}$] (i.e., 1/16 of the expected offspring assuming a Mendelian distribution) has been lower than the other 3 genotypes, suggesting a reduced embryonic or perinatal viability in the absence of the $A_1$ receptor in this mouse strain (C57Bl/6). To simplify the breeding and improve the yield we may cross only $A_1$ heterozygotes to assess the role of the just $A_1$ receptor in a neurotoxin model of PD (separately from that of the $A_{2A}$ receptor, which we previously established).

*Exp# 2* – **Effect of brain-specific $A_{2A}$ KO in MPTP and Pq/Mb models**.

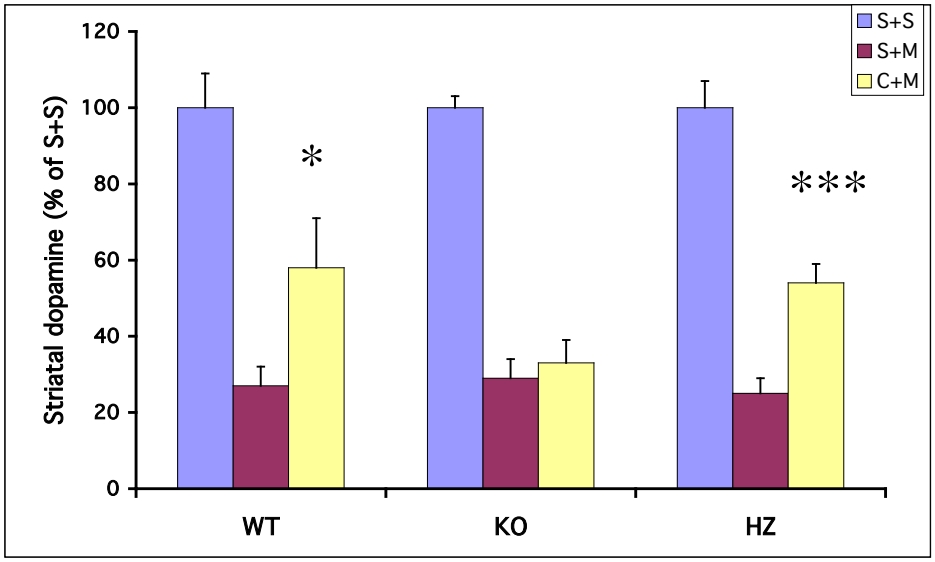
As reported for Year 1, we completed the generation and initial characterization of a conditional (Cre/*loxP* system) KO of post-natal forebrain neuronal $A_{2A}$ receptors. The *CamKII*$\alpha$ promoter was used to drive expression of the *cre* recombinase gene in postnatal forebrain neurons, and thus to cause selective depletion of striatal neuron $A_{2A}$ receptors following brain development. We recently published (Bastia et al, 2005[6]) evidence of successful forebrain-specific recombination by genetic, autoradiographic and behavioral assessments.

In preliminary experimentes, we found no consistent phenotype of the forebrain neuron $A_{2A}$ receptor (conditional) KO on its own with respect to MPTP toxicity (see below). This allowed us to ask whether these conditional 'brain-specific' $A_{2A}$ (conditional) KO show a reduction in caffeine's neuroprotective effect in this PD model. (See Exp #3 below.)

*Exp# 3* – **Brain $A_{2A}$R-dependence of caffeine's neuroprotective effect**.

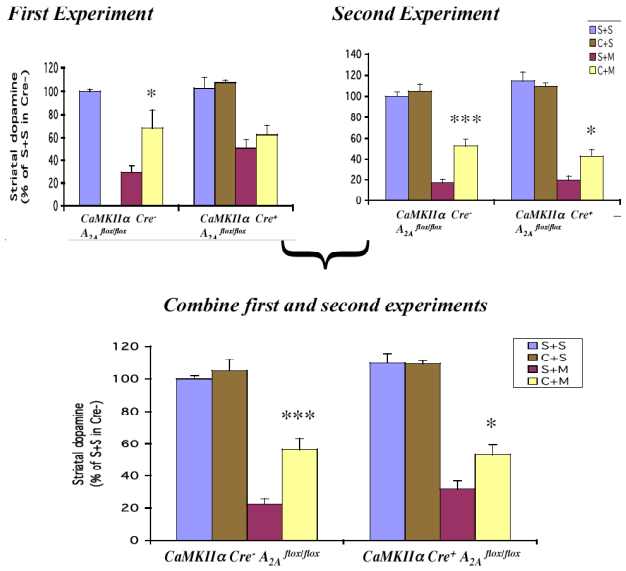
Leading up to this experiment we found that the complete loss of neuroprotection by caffeine in global $A_{2A}$R KO mice establishes the adenosine $A_{2A}$R as a critical mediator of caffeine's neuroprotective effects in this model of Parkinson's disease. See Fig. 1. By contrast, caffeine maintains the ability to at least partially protect against MPTP toxicity in conditional KOs of forebrain neuron $A_{2A}$Rs (Fig. 2) and of astrocyte (Fig. 3) $A_{2A}$Rs. Together the findings suggest that the neuroprotective actions caffeine in this PD model depend at least partially on $A_{2A}$Rs other than those located on forebrain (striatal and cortical) neurons and on astrocytes.

## *Fig. 1.* Caffeine attenuated MPTP-induced striatal dopamine loss in WT and HZ, but not global $A_{2A}$R KO male mice.



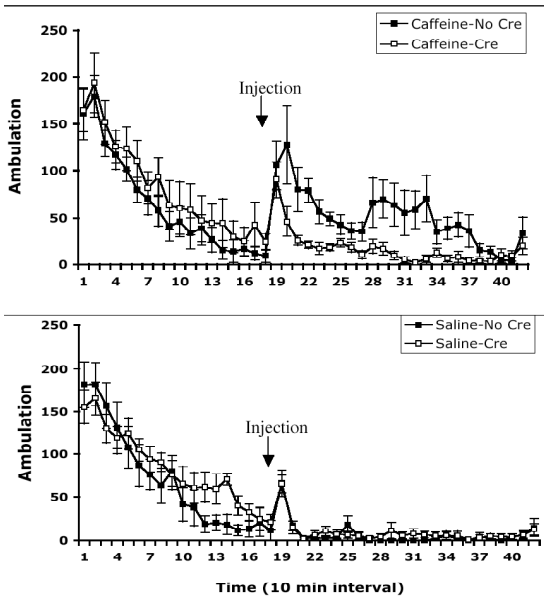

N=3 for saline treatments and n=6-9 for MPTP treatments. *S*, saline; *M*, MPTP (35 mg/kg ip single injection); *C*, Caffeine (25 mg/kg ip 10min before MPTP). *p<0.05, ***p<0.001 compared with respective *S+M* group.

***Fig 2.*** ***A.*** **Caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal $A_{2A}$Rs.**



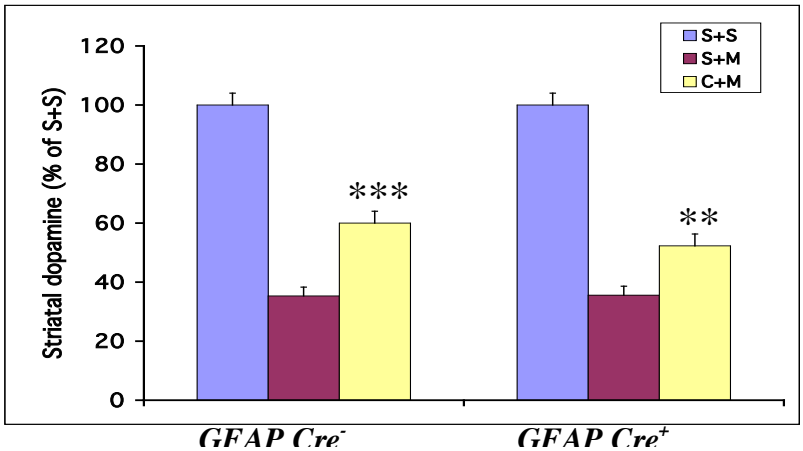

N=3-4 for saline treatments and n=3-13 for MPTP treatments in either first or second experiment. *p<0.05, ***p<0.001 compared with respective *S+M* group.

***B.*** **Locomotion after *caffeine* injection is significantly reduced in forebrain neuron $A_{2A}$R cKO (*CaMKIIα Cre$^+$ $A_{2A}^{flox/flox}$*) than that in control (*CaMKIIα Cre$^-$ $A_{2A}^{flox/flox}$*) male mice, while locomotions after *saline* injections are similar in $A_{2A}$R cKO and control mice.**



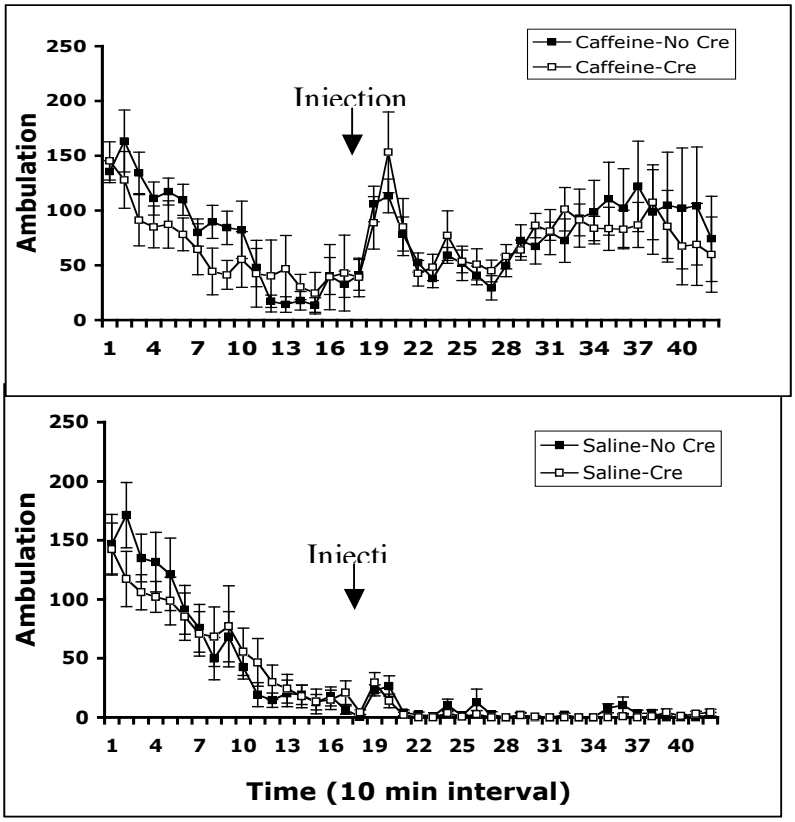

N=8 for each group. The dose of caffeine is 25 mg/kg.

***Fig. 3A.*** **Caffeine attenuated MPTP-induced striatal dopamine loss in control (*GFAP Cre$^-$ $A_{2A}^{flox/flox}$*) and <u>astrocyte-directed</u> $A_{2A}$R cKO (*GFAP Cre$^+$ $A_{2A}^{flox/flox}$*) mice.**




N=6-7 for saline treatments and n=22-39 for MPTP treatments. **p<0.01, ***p<0.001 compared with respective *S+M* group.

***3B.*** **Locomotions after *caffeine* or *saline* injections are similar in control (*GFAP Cre$^-$ $A_{2A}^{flox/flox}$*) and <u>astrocyte-directed</u> $A_{2A}$R cKO (*GFAP Cre$^+$ $A_{2A}^{flox/flox}$*) mice.**




N=8 for each group. The dose of caffeine is 25 mg/kg.

# *Methods (for Figs. 1-3)*

**Animals** Global $A_{2A}R$ KO mice were generated using a standard displacement target vector. Postnatal forebrain neuron- and astrocyte-directed $A_{2A}$ cKO mice were generated using the Cre-*loxP* system based on the specificities of *CaMKIIα* and *GFAP* gene promoters, respectively. Tissue-specific disruption of the $A_{2A}R$ was confirmed by PCR and western blot. Congenic (N10, C57Bl/6 background) global $A_{2A}R$ KO, wide-type (WT) and heterozygous (HZ) mice, and near congenic (N6, C57Bl/6) $A_{2A}^{flox/flox}$ mice (i.e., homozygous for the floxed allele of the $A_{2A}R$ gene) with or without a *cre* transgene were used for this study. A *cre* transgene was introduced by cross-breeding to either a *CaMKIIα-cre* or *GFAP-cre* lines.

**MPTP-induced dopaminergic neurotoxicity.** Caffeine (25 mg/kg) or saline were injected intraperitoneally (ip) 10 minutes before MPTP-HCl treatment (35 mg/kg ip single injection). One week after MPTP treatment, the striata were dissected and analyzed for dopamine content using standard reverse-phase HPLC with electrochemical detection. Dopamine content was calculated as pmol/mg of tissue and values are presented within the figures as percent change from respective Saline-Saline treated controls.

**Caffeine-stimulated locomotion.** Mice were habituated in the testing cages and basal spontaneous locomotion was recorded for at least 180 min. Then locomotor behavior was monitored for another 240 min after caffeine (25 mg/kg ip) or saline injection. Locomotion (*Ambulation*) was scored as the number of adjacent photobeam breaks.

**Statistical analyses.** The data from striatal dopamine content as well as caffeine-stimulated locomotion were analyzed by two-way ANOVA. Post-hoc comparisons were performed using Bonferroni test. Data values in the figures represent group mean ± SEM.

***Specific Aim #2*** – to localize the region within brain through which caffeine or $A_{2A}$ receptor inactivation produces its neuroprotective effect in the MPTP model of PD. **(~576 mice)**

**[Please see abstract publications in Appendices B and C.]**

*Hypothesis 2:* **Caffeine perfusion and focal $A_{2A}$ receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

*Exp# 4* – **Effect of intracerebral caffeine perfusion on MPTP-induced neurotransmitter overflow and toxicity**:

In preparation for this experiment with local administration of caffeine, we are continuing to characterize (using microdialysis) MPTP-induced neurotransmitter overflow in the striatum and its modulation by systemic caffeine.

*Exp# 5* – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed $A_{2A}$R mice**: Homozygous floxed $A_{2A}$R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP. One week later infusion needle track will be localized histochemically, while dopaminergic neuron integrity will be visualized by striatal DAT binding and nigral TH-IR counts will be assessed as in Exp #4.

During Year 2 we have made further progress toward this experiment in characterizing the viral Cre/*loxP* conditional KO method that was adopted in Year 1. We have demonstrated that the resulting unilateral local recombination and disruption of the $A_{2A}$ gene, and in turn the elimination of striatal $A_{2A}$ receptors are dose-(titer-)dependent and occur sequentially. Working with several serotypes of AAV-*cre* and AAV-*GFP* (provided through a collaboration with Dr. Miguel Esteves), Dr. Augusta Pisanu has now demonstrated that:

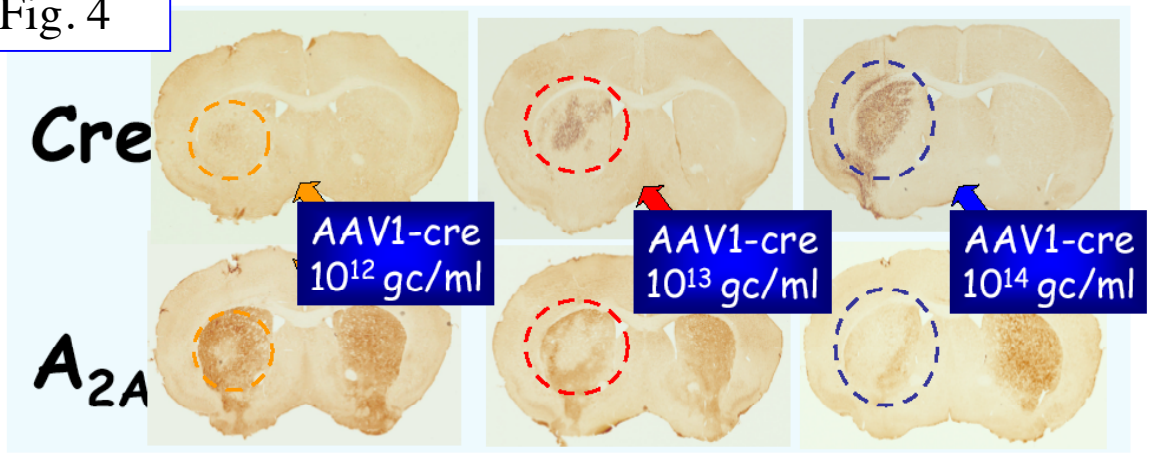
- Using this AAV-Cre/*lox*P system we were able to obtain a conditional KO of the $A_{2A}$R in the striatum of adult mice. Whereas the Cre expression was detectable within 8 days after injection (Fig. 5B), the $A_{2A}$R-IR loss was not evident until the 16$^{th}$ day (Fig. 5D), presumably due to the stability of the already expressed receptors, and reached a maximum level by the 32$^{nd}$ day post injection. (Figs. 4 and 5D.)

- This viral *cre*-based Cre/*lox*P conditional KO of the $A_{2A}$R was also a titer-dependent phenomenon since an increasing viral titer produces a greater the number of infected cells and further reductions in $A_{2A}$R-IR. (Figs. 4 and 5A and C.)

- The viral vector titer not only determines the intensity of $A_{2A}R$ KO, but also the spatial extent of the infection, and consequently the $A_{2A}R$ loss (Fig. 4), suggesting that the virus particles may diffuse through the brain parenchyma until they find a "free" binding site that stops their diffusion and let them into a host cell. Is it important to consider whether an excessive viral concentration could produce an over-expression of Cre, which is capable of inducing cell toxicity (as detected in the center of the infected area in some mice infected with the highest AAV-cre titer, data not shown).

- In this model, the AAV-Cre/*loxP* conditional KO system provides a temporally and spatially controlled method to precisely eliminate $A_{2A}R$ in discrete brain regions providing additional advantages in exploring the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

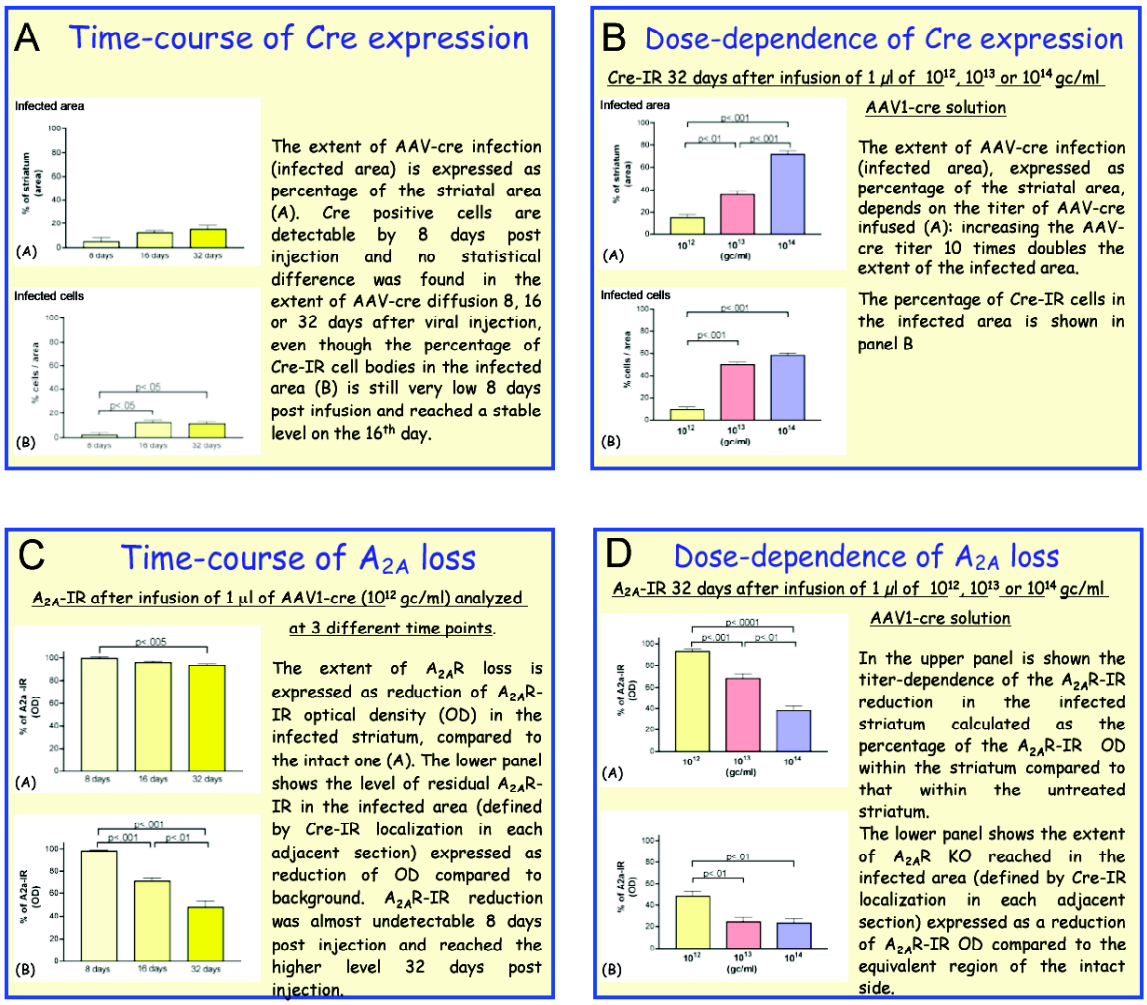
Fig. 4




This methodological advance will allow us to dissect $A_{2A}$ receptor involvement in neurotoxin models of PD with an unprecedented combination of anatomical and molecular precision. We are now initiating the MPTP studies in these mice lacking $A_{2A}$ receptors in discrete brain regions.

*

Fig. 5




# Methods for Figs. 4 and 5

Mice with a *floxed* $A_{2A}R$ gene were generated by insertion of *lox*P sequences within the introns flanking the exon 2 of the $A_{2A}R$ gene (YJ Day and J linden, unpublished results). Homozygous adult male mice were anesthetized with an i.p. injection of Avertin and positioned in a stereotaxic frame for injection into the left striatum (AP: +0.7; ML: +2; DV: -2.8) of 1 $\mu$l of AAV1-cre ($9.3x10^{13}$, $1x10^{13}$ or $1x10^{12}$ gc/ml) at a rate of 0.1 $\mu$l/min by using a 30 gauge needle connected to a 50$\mu$l Hamilton syringe driven by a microinfusion pump.

After 8, 16 or 32 days mice were anesthetized and intracardially perfused 10 ml of ice-cold saline followed by 50 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer. After perfusion brains were removed and incubated overnight in

the same fixative than cryoprotected by incubation in phosphate buffered 30% sucrose. Serial 25 $\mu$m-thick coronal sections were cut on a freezing microtome and collected in 50 mM Tris buffer. Adjacent sections starting from the rostral part of the striatum were collected for immunohistochemical staining for Cre recombinase (1:2000 Novagen anti-Cre rabbit polyclonal antibody) and $A_{2A}R$ (1:200 Santa Cruz anti A2AR goat polyclonal antibody). Donkey anti-species antibodies conjugated to biotin, Vectastain ABC Kit and fast DAB Kit were used for detecting primary antibodies. Immunostainig controls were done without the primary antibody. Quantification of Cre and A2aR-immunoreactivity (IR) was performed every sixth section at 10X and 40X magnification. Statistical analysis was performed by Oneway ANOVA followed by a Tukey post hoc analysis of means difference between groups.

***Specific Aim #3*** – to investigate caffeine-estrogen interactions in the MPTP model of PD by determining the effect estrogen replacement on the neuroprotective phenotype of $A_{2A}$ KO mice, and exploring potential peripheral and CNS mechanisms contributing to caffeine's reduced neuroprotective efficacy in the presence of estrogen.

**[Please see Appendix N.]**

With support from this award we have now published our studies of estrogen-caffeine interaction in the MPTP model of Parkinson's disease, as detailed in the attached final manuscript (Appendix A). Our results demonstrate that estrogen reduces caffeine's neuroprotective effect against MPTP toxicity in both male and female mice. In the context of human epidemiology on PD, our findings suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

# Body of the Report: from Yr 3 Annual Report

Progress during Year 3 on Specific Aims and experiments as laid out in our Statement of Work (SOW [in blue]) is described here in detail.

## STATEMENT OF WORK (focus on main areas of progress in Yr 3)

***Specific Aim #1*** – to definitively determine whether brain $A_{2A}$Rs or $A_1$Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain $A_{2A}$R is required for caffeine's protective effect in these PD models.

**[Please see Appendix R.]**

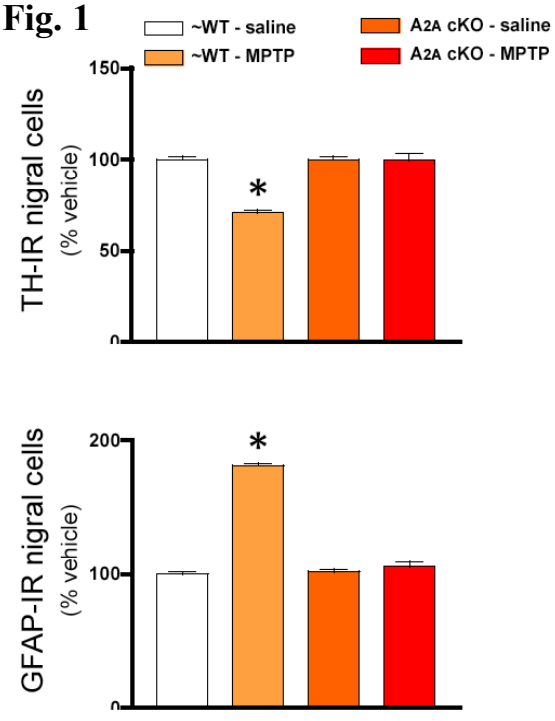
*Hypothesis 1:* **Caffeine acts through blockade of brain $A_{2A}$ (not $A_1$) receptors to protect dopaminergic neurons in both acute (MPTP) and chronic (paraquat/maneb) toxin models of PD.**

*Exp# 2* – **Effect of brain-specific $A_{2A}$ KO in MPTP and Pq/Mb models**.

As reported for Year 1, we completed the generation and initial characterization of a conditional (Cre/*loxP* system) KO of post-natal forebrain neuronal $A_{2A}$ receptors. The *CamKIIα* promoter was used to drive expression of the *cre* recombinase gene in postnatal forebrain neurons, and thus to cause selective depletion of striatal neuron $A_{2A}$ receptors following brain development. We published (Bastia et al, 2005[6]) evidence of successful forebrain-specific recombination by genetic, autoradiographic and behavioral assessments. We reported that during project Yr 2 that we found no consistent phenotype of the forebrain neuron $A_{2A}$ receptor conditional KO (cKO) with respect to *acute* MPTP toxicity (see below).

In contrast, during Yr 3 we have found substantial attenuation of dopaminergic neuron injury in the forebrain neuron $A_{2A}$ cKO mice in a more *subacute* toxin exposure (multiple smaller doses of MPTP administered over days) – potentially of greater pathophysiological relevance than the the acute/single high dose MPTP paradigm. In experiments conducted in collaboration with the laboratory of Prof. Micaela Morelli of the Univ. of Cagliari we found (**Fig. 1**) that the loss of doapminergic (TH-IR) nigral neurons and the accompanying astrogliosis (GFAP-IR cell increase) was completely absent in littermate forebrain neuron $A_{2A}$ cKO mice (i.e., mice homozygous

**Fig. 1**

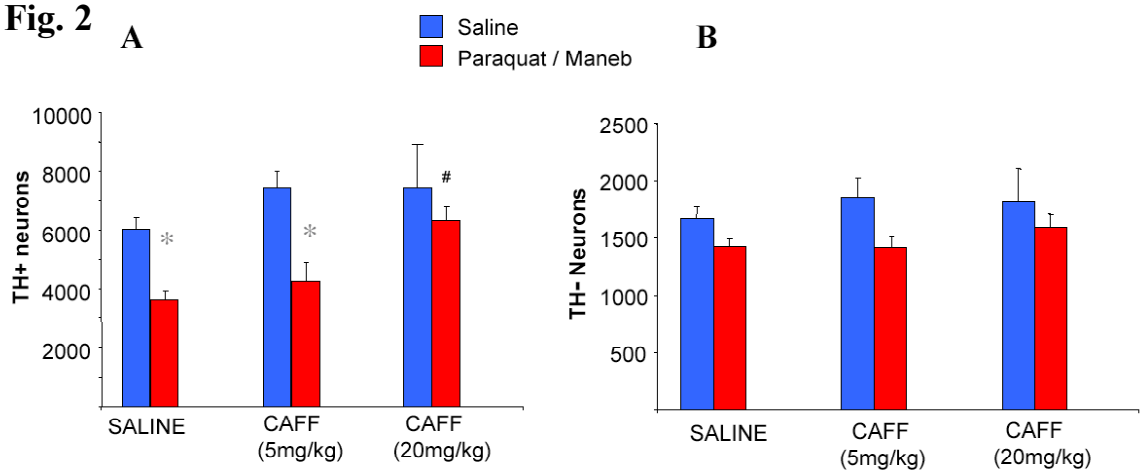


for the *floxed* $A_{2A}$ gene and also transgenic for the *cre* gene.

*Exp# 3* – **Brain $A_{2A}$R-dependence of caffeine's neuroprotective effect**.

To explore this possibility in a chronic dual pesticide (paraquat + maneb) model of PD we first had to determine whether caffeine is protective in this model. C57Bl/6 mice were treated twice weekly with paraquat (Pq) and maneb (Mb) i.p. for 8 weeks. Ten min prior to each Pq+Mb toxin administration mice were pretreated with caffeine at 0 (saline vehicle), 5 or 20 mg/kg. Caffeine at the moderate dose of 20 mg/kg significantly attenuated the pesticide-induced loss of dopaminergic (TH+) nigral neurons, assessed by rigorous stereological methods. (See **Fig. 2A** and **Appendix X**.) By contrast, caffeine had no effect on TH- (non-TH immunoreactive) nigral cells (**Fig. 2B**), confirming the neuroprotective effect of caffeine (i.e., by excluding an induction of TH-immunorectivity in originally TH- cells masking a loss of originally TH+ neurons in the mice pretreated with 20 mg/kg caffeine).

**Fig. 2**




In Yr 3 we also initiated a study of the neuroprotective potential of urate, the end product of adenosine metabolism in humans, the immediate oxidation of xanthine, and a structural analog of caffeine (a.k.a. tri-methyl-xanthine). Urate is also a major antioxidant and is the main circulating antioxidant in humans in whom it circulates near the limits of solubility (and hence its pathophysiological role in urate crystal diseases like gout). Based on its antioxidant and chelating properties we and others have found urate to be the first molecular predictor of both risk of PD and the rate at which it progresses. (See **Appendices Q** and **U** for furhter background.) We have provided the first evidence that urate can be neuroprotective in an *in vivo* model of PD (see **Appendix H**) by showing that locally administered (intracerebroventricular) urate can attenuate the toxicity of systemic MPTP.

***Specific Aim #2*** – to localize the region within brain through which caffeine or $A_{2A}$ receptor inactivation produces its neuroprotective effect in the MPTP model of PD.

**[Please see abstract publication in Appendix G.]**

*Hypothesis 2:* **Caffeine perfusion and focal $A_{2A}$ receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

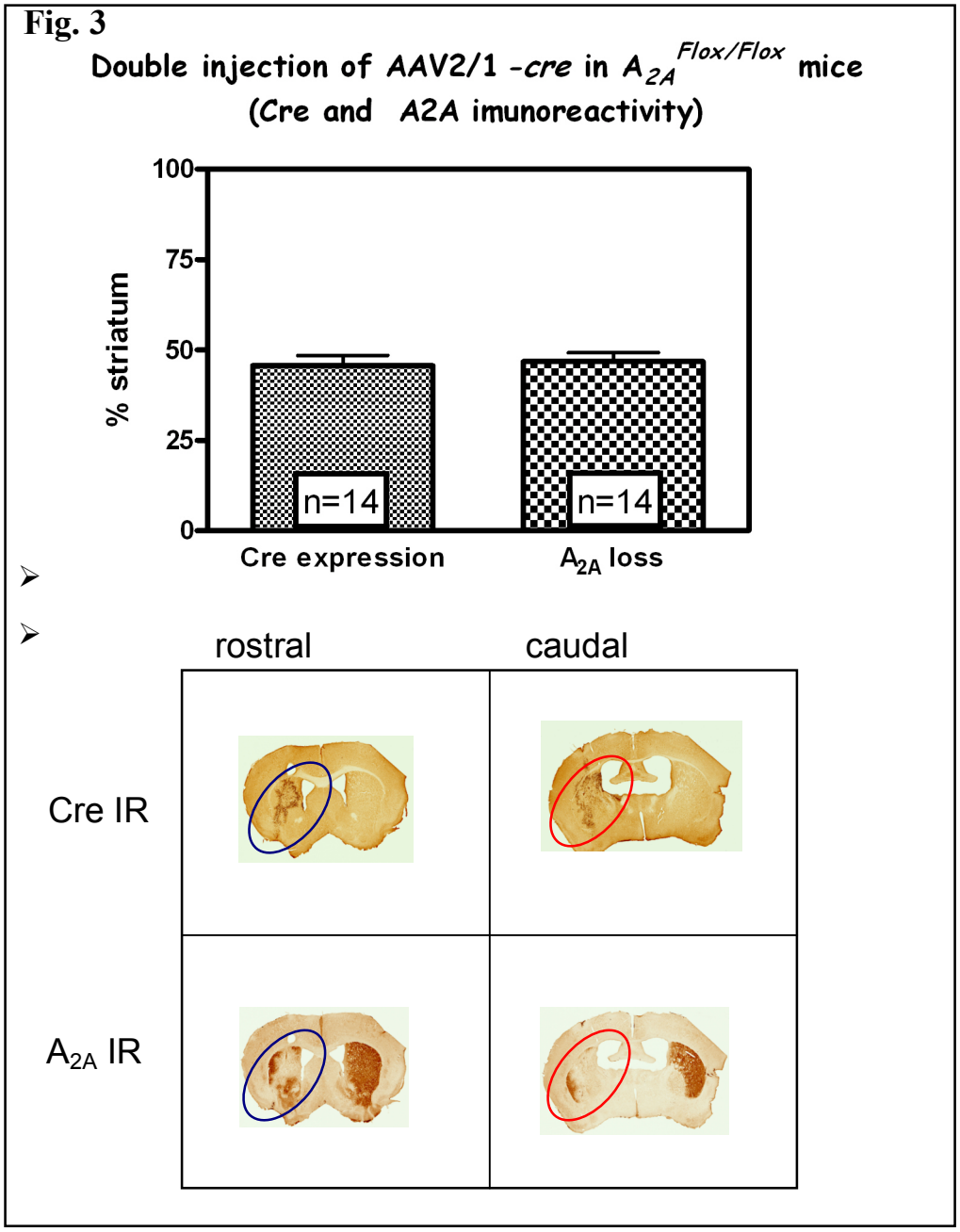
*Exp# 5* – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed $A_{2A}$R mice**: Homozygous floxed $A_{2A}$R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP. One week later infusion needle track will be localized histochemically, while dopaminergic neuron integrity will be visualized by striatal DAT binding and nigral TH-IR counts will be assessed as in Exp #4.

As reported in Yr 2 we have characterized the time course and the dose-(titer-) dependence of unilateral local recombination and subsequent disruption of the $A_{2A}$ gene in the viral Cre/*loxP* conditional KO mothod we adopted.

During Yr 3 we have further characterized the phenotype of this $A_{2A}$R cKO in the striatum of adult mice by testing the effect of unilateral striatal $A_{2A}$R loss in turning behavior and MPTP-induced loss of striatonigral dopaminergic neurons. Working with AAV2/1-*cre* and AAV2/1-*GFP* viruses (provided through a collaboration with Dr. Miguel Sena-Esteves of our institution), Dr. Augusta Pisanu has now demonstrated that:

- Using this AAV-Cre/*loxP* system, a double injection of AAV2/1-*cre* ($3x10^{13}$ gc/ml) into the rostral and caudal striatum induces 50% of loss of $A_{2A}$ striatal receptors in $A_{2A}^{flox/flox}$ mice (**Fig 3**). This focal $A_{2A}$R KO phenotype was reached 30 days post injection and was still stable until 60 days post injection, although at that time cell toxicity was detected in the center of the infected area in some mice, probably related to an over-expression of Cre across time. (To reduce this late cell toxicity, in collaboration with Dr. Esteves, we are now testing a new and promising AVV vector, which delivers the Cre recombinase gene under control of a weaker promoter with the goal of eliminating toxicity.)

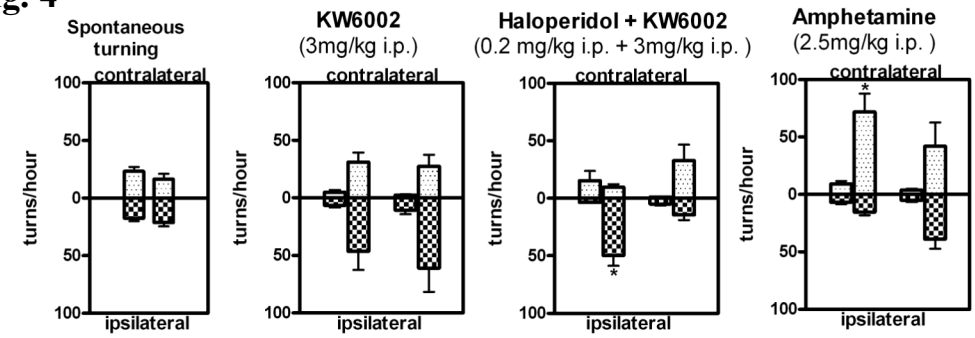
**Fig. 3**




- This degree of unilateral loss of $A_{2A}R$ in mouse striatum is sufficient to induce a behavioral phenotype. Starting from the 30th day, to assess for asymmetries in striatal control of movement, turning behaviour was monitored in unilateral cKO mice (AAV2/1-*cre*, $A_{2A}R^{flox/flox}$) and in control mice (AAV2/1-*GFP*, $A_{2A}R^{flox/flox}$). None of the experimental groups showed a biased spontaneous turning behaviour. In unilateral conditional KO mice the $A_{2A}$ antagonist KW-6002 (3 mg/kg, ip; in the presence of a sub-threshold dose of the dopamine $D_2$ antagonist haloperidol) induced ipsilateral turning, and amphetamine (2.5 mg/kg i.p.

on its own) induced contralateral turning relative to that in the control animals (**Fig. 4**).

**Fig. 4**




- AAV2/1-*cre* injection into the striatum of $A_{2A}R^{flox/flox}$ mice confered a partial but significant neuroprotection against acute MPTP intoxication. 50 days after infection AAV2/1-*cre* injected mice and their respective AAV2/1-*GFP* controls were treated with MPTP or saline (4 times 15mg/kg i.p every 2 hours). Seven days after, the TH immunoreactivity of sparing projections in the striatum was measured: the AAV2/1-*cre*, $A_{2A}R^{flox/flox}$ mice showed a more intense TH staining in the infected side, compared to the intact one, while in the AAV2/1-*GFP*, $A_{2A}R^{flox/flox}$ group no difference was detectable (**Figs. 5 and 6**). Further stereological analysis of sparing neurons in *substantia nigra pars compacta* is now in progress, to confirm that focal disruption of $A_{2A}$ receptor in the striatum is able to confer neuroprotection to dopaminergic neurons in the MPTP model of PD.

- **Fig. 5**

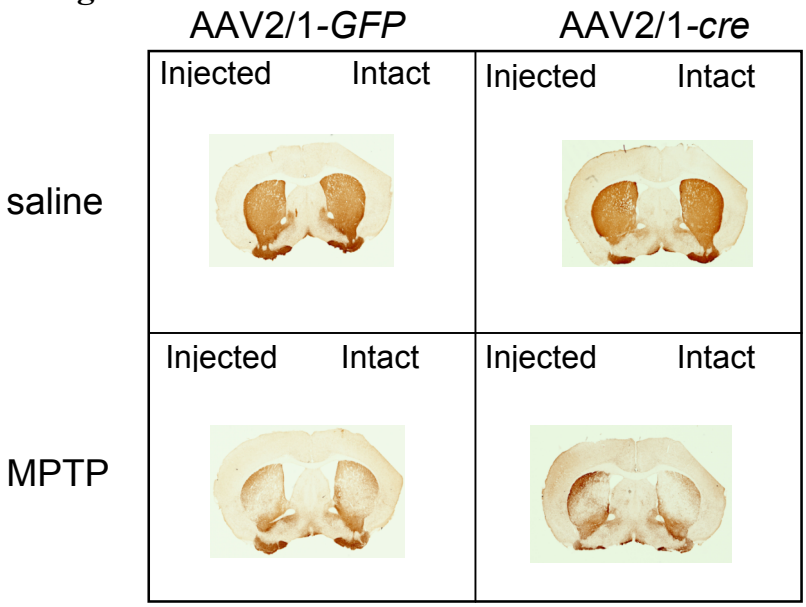
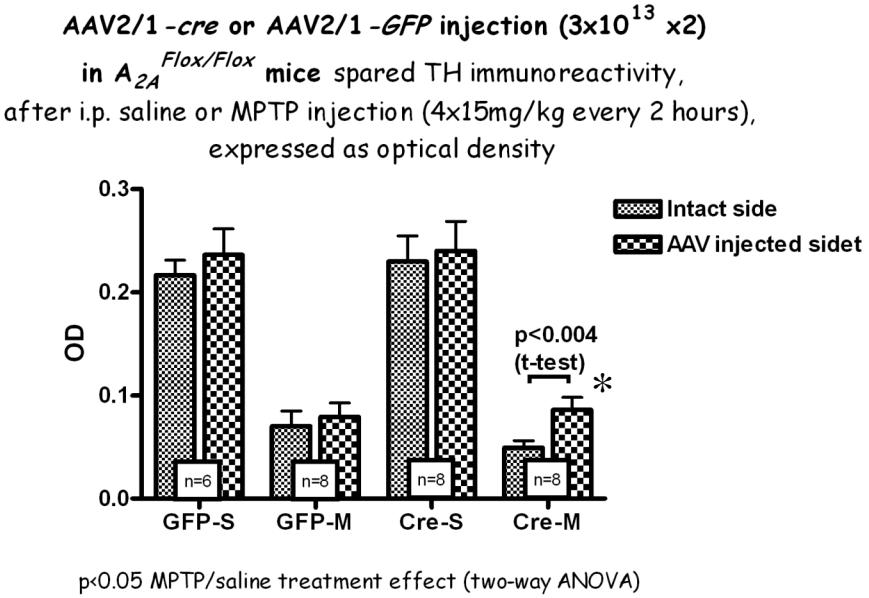


**Fig. 6**




- This methodological advance will allow us to dissect $A_{2A}$ receptor involvement in neurotoxin models of PD with an unprecedented combination of anatomical and molecular precision in different brain structures.

# Methods (for Figs. 3-6)

Mice with a *floxed* $A_{2A}R$ gene were generated by insertion of *lox*P sequences within the introns flanking the exon 2 of the $A_{2A}R$ gene (YJ Day and J linden, unpublished results). Homozygous adult male mice were anesthetized with an i.p. injection of Avertin and positioned in a stereotaxic frame for double injection into the left striatum (rostral: AP: +1.2; ML: +1.5; DV: -2.75; caudal: AP: -0.15; ML: +2.5; DV: -2.65) of 1 $\mu$l of AAV2/1-*cre* or AAV2/1-*GFP* ($3x10^{13}$ gc/ml) at a rate of 0.1 $\mu$l/min by using a 30 gauge needle connected to a 50$\mu$l Hamilton syringe driven by a microinfusion pump.

After 30 days mice were tested for asymmetries in striatal control of movement by recording contralateral and ipsilateral turns during the dark phase of their circadian rhythm, using an automated rotometry system (San Diego Instruments, San Diego, CA). Each mouse was placed at the center of 1 of 12 opaque glass flat-bottom bowls (10 cm diameter base, 13 cm high wall, with opening diameter of 26 cm) and connected to the lower end of a customized cable tether by a rubber band snugly fitted around the chest. The upper end of the cable is attached to a swivel box, which in turn is linked to a computer interface.

The $50^{th}$ day the animals were randomly divided in 4 groups and received MPTP or saline injections (4 time 15mg/kg i.p. MPTP-HCL or saline every 2 hour) and 7 days later they were anesthetized and intracardially perfused with 10

ml of ice-cold saline followed by 30 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer. After perfusion brains were removed and incubated overnight in the same fixative than cryoprotected by incubation in phosphate buffered 30% sucrose. Serial 25 $\mu$m-thick coronal sections were cut on a freezing microtome and collected in 50 mM Tris buffer. Adjacent sections starting from the rostral part of the Striatum to the Sunstantia Nigra pars compacta (Snc) were collected for immunohistochemical staining for Cre recombinase (1:2000 Novagen anti-Cre rabbit polyclonal antibody) $A_{2A}R$ (1:200 Santa Cruz anti $A_{2A}R$ goat polyclonal antibody) and Tyrosine Hydroxylase(1:1000 Biomol anti-TH rabbit polyclonal antibody). Goat or Horse anti-species antibodies conjugated to biotin, Vectastain ABC Kit and fast DAB Kit were used for detecting primary antibodies. Immunostainig controls were done without the primary antibody. Quantification of Cre and A2aR-immunoreactivity (IR) was performed every sixth section at 4X magnification by measuring the extent of the immunopositive or immunonegative area in the Striatum. TH-immunoreactivity was quantified every 12 sections at 4X magnification by measuring the Optical Density (OD) of the striatal area, corrected by the OD of overlying cortex. as background. Statistical analysis was performed by Oneway ANOVA followed by a Tukey post hoc analysis of means difference between groups.Student's t test was used for the remaining statistical analyses. A value of p  0.05 was considered to be significant.

***Specific Aim #3*** – to investigate caffeine-estrogen interactions in the MPTP model of PD by determining the effect estrogen replacement on the neuroprotective phenotype of $A_{2A}$ KO mice, and exploring potential peripheral and CNS mechanisms contributing to caffeine's reduced neuroprotective efficacy in the presence of estrogen.

**[Please see publication in Appendix O, a translational neuroscience review focused on developing neuroprotective therapeutics for PD, with caffeine and estrogen included in the context of broader strategies.]**

With support from this award we have now fully completed and published our studies of estrogen-caffeine interaction in the MPTP model of Parkinson's disease, as detailed in our Yr 2 progress reported. Our results demonstrate that estrogen reduces caffeine's neuroprotective effect against MPTP toxicity in both male and female mice. In the context of human epidemiology on PD, our findings suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

# Body of the Report: from Yr 4 Annual Report

Progress during Year 4 on Specific Aims and experiments as laid out in our original Statement of Work (SOW [in blue]) is described here in detail.

## STATEMENT OF WORK (focus on main areas of progress in Yr 4)

***Specific Aim #1*** – to definitively determine whether brain $A_{2A}$Rs or $A_1$Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain $A_{2A}$R is required for caffeine's protective effect in these PD models.

**[Please see manuscript and abstract publication in Appendices I and X.]**

- Completion of work demonstrating that the non-specific adenosine antagonist caffeine can in fact confer neuroprotection in a chonic dual pesticide toxin model of PD. Pretreatment with caffeine attenuated loss of nigral dopaminergic neurons in mice chronically exposed (twice weekly for two months) to paraquat plus maneb. Rigorous stereological assessments were made of tyrosine hydroxylase-immunoreactive cells to determine total dopaminergic neuron counts of the substantia nigra. A manuscript reporting these findings has now been submitted for publications (Appendix X).
- In Year 4 we have also investigated and preliminarily reported (Appendix I) how depletion of adenonsine $A_{2A}$ receptors (in $A_{2A}$ knockout mice) may mimic the neuroportective effect of caffeine in a chronic mouse model of PD. Here we used an lifelong exposure to an 'endogenous toxin' of transgenically expressed mutant human α–synuclein (*hw-αSYN*). Mutations in the human α–synuclein gene (A53T and A30P) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human α –synuclein gene with both of these mutations have been shown to develop age-related reductions in dopaminergic nigral neuron numbers, striatal dopamine (DA) and motor activity.

  We investigated whether deletion of the $A_{2A}$ receptor (in $A_{2A}$[-/-] mice) would afford protection against the age-related spontaneous reduction of striatal dopamine in the double-mutant human α -synuclein (*hm²-αSYN*) mice. Heterozygous $A_{2A}$[+/-] mice were mated with $A_{2A}$[+/-] mice that were also transgenic for the wild-type (*hw-αSYN*) or *hm²-αSYN* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter – all in a congenic C57Bl/6 background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional $A_{2A}$ receptor, DA content was significantly lower (by 35%) in hm²-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in $A_{2A}$[-/-] mice (lacking functional $A_{2A}$ receptor) the hm²-αSYN transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender. Stereological nigral TH-IR cell counts are in progress. Thus the $A_{2A}$ receptor appears to be required for neurotoxicity in an α–synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific $A_{2A}$ antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

***Specific Aim #2*** – to localize the region within brain through which caffeine or $A_{2A}$ receptor inactivation produces its neuroprotective effect in the MPTP model of PD.

[Please see abstract publication in Appendix J.]

*Hypothesis 2:* **Caffeine perfusion and focal $A_{2A}$ receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

*Exp# 5* – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed $A_{2A}$R mice**: Homozygous floxed $A_{2A}$R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP...

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Progress in Year 4 has been focused on working through technical limitations of potential AAV toxicity confounding interpretation of the hypothesized protective effect conferred by the Cre-medicated recombination event produced by viral expression of *cre* in the striatum. As reported in Year 3 (see Progress Report Yr 3), using the AAV-Cre/*lox*P system, with a double injection of AAV2/1-*cre* ($3x10^{13}$ gc/ml), in the rostral and caudal striatum respectively, we demonstrated the induction of a 50% loss of $A_{2A}$ striatal receptors in $A_{2A}^{flox/flox}$ mice. This focal $A_{2A}$R KO was still stable until 60 days post injection and was sufficient to induce a behavioral phenotype and to confer partial but significant neuroprotection against acute MPTP intoxication (see Appendix J). On the other hand at that time (60 days post injection) cell toxicity was detected in the center of the infected area in some mice, probably related to an over-expression of Cre recombinase across time. To reduce this late cell toxicity in collaboration with Dr. Miguel Sena-Esteves at our institution, we tested a promising alternative AVV vector, which delivers the Cre recombinase under control of a weaker promoter: the rat synapsin 1 promoter, that should confer a higher neuronal specificity for the infection and reduce the amount of Cre expressed in every single cell (and, we reasoned, should reduce the toxicity accordingly).

Although AAV2/1-syn1-*cre* ($1x10^{13}$ gc/ml and $6x10^{13}$ gc/ml) injection in the striatum of $A_{2A}R^{flox/flox}$ mice did not induce a Cre expression detectable with IHC techniques (as we expected because of the weaker promoter), it did not induce appreciable loss of $A_{2A}$R-IR loss in the brain tissue surrounding the injection area. Nevertheless, a further analysis of the targeted neurons revealed also a loss of DARPP-32-IR (a marker for srtiatal neurons, which express high levels of this protein) in the same area. Thus, it appears attempt to obviate this technical limitation was not successful, and we have returned to the successful original promoter AAV construct and will pursue alternative strategies to minimize it's long-term toxicity.

*Detailed method*: Mice with a *floxed* $A_{2A}R$ gene were generated by insertion of *lox*P sequences within the introns flanking the exon 2 of the $A_{2A}R$ gene (YJ Day and J linden, unpublished results). Homozygous adult male mice were anesthetized with an i.p. injection of Avertin and positioned in a stereotaxic frame for double injection into the left striatum (rostral: AP: +1.2; ML: +1.5; DV: -2.75; caudal: AP: -0.15; ML: +2.5; DV: -2.65) of 1 $\mu$l of AAV2/1syn1-*cre* ($1x10^{13}$ and $6x10^{13}$ gc/ml) at a rate of 0.1 $\mu$l/min by using a 30 gauge needle connected to a 50$\mu$l Hamilton syringe driven by a microinfusion pump.

After 16, 30 and 60 mice were anesthetized and intracardially perfused with 10 ml of ice-cold saline followed by 30 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer. After perfusion brains were removed and incubated overnight in the same fixative than cryoprotected by incubation in phosphate buffered 30% sucrose. Serial 35 $\mu$m-thick coronal sections were cut on a freezing microtome and collected in 50 mM Tris buffer. Adjacent sections starting from the rostral part of the Striatum to the Sunstantia Nigra pars compacta (Snc) were collected for immunohistochemical staining for Cre recombinase (1:2000 Novagen anti-Cre rabbit polyclonal antibody) $A_{2A}R$ (1:200 Santa Cruz anti $A_{2A}R$ goat polyclonal antibody) and DARPP-32 (1:1000 Cell Signaling anti-DARPP-32 rabbit polyclonal antibody). Goat or Horse anti-species antibodies conjugated to biotin, Vectastain ABC Kit and fast DAB Kit were used for detecting primary antibodies. Immunostainig controls were done without the primary antibody. Quantification of Cre, A2aR and DARPP32-immunoreactivity (IR) was performed every sixth section at 4X magnification by measuring the extent of the immunopositive or immunonegative area in the striatum.

# Body of the Report: from Yr 5 Annual Report

Progress during Year 5 on Specific Aims and experiments as laid out in our approved modification of the Statement of Work (SOW [in blue]) is described here in detail.

## STATEMENT OF WORK (focus on main areas of progress in Yr 5)

***Specific Aim M1:*** to definitively determine whether brain $A_{2A}$Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD.

**[Please see manuscript and abstract publication in Appendix K.]**

- We have obtained and now published (Appendix K) evidence that forebrain $A_{2A}$ receptors are in fact required for dopaminergic neuron toxicity in a chronic toxin model of PD. Working with collaborators at the University of Cagliari we have compared the dopaminergic neuron toxicity produced by repeated daily administration of MPTP toxin in mice in which the adenosine $A_{2A}$ receptor is conditionally 'knocked out' (cKO) of postnatal forebrain (striatal and cortical) **neurons**, compared to their control littermates with normal $A_{2A}$ receptor expression. The findings attenuated toxicity and inflammation support the possibility that specific adenosine $A_{2A}$ receptors -- currently in clinical (phase II/III) development as symptomatic therapy for later stage PD patients -- may have disease-modifying (neuroprotective) benefits if admininistered in earlier in the course of PD.

  Using the same Cre/*loxP* cKO system, we similarly compared the toxicity produced by repeated daily administration of MPTP toxin in mice in which the adenosine $A_{2A}$ receptor is conditionally 'knocked out' of **astrocytes** compared to their control littermates with normal $A_{2A}$ receptor expression. The finding that these astrocytic $A_{2A}$ receptor cKO mice were not protected as observed in the neuronal $A_{2A}$ cKO, suggest neuronal $A_{2A}$ receptors could fully account for the hypothesized benefits of $A_{2A}$ antagonists against neurodegeneration in PD. The astrocyte $A_{2A}$ cKO data were removed from the final published manuscript pending a more complete validation of this cKO reagent (e.g., showning specific delepletion of A2A receptors from astrocytes in this line, given that our findings with this line showed no phenotype in this PD model).

- Also in Year 5, and in pursuit of the original SA #1, we have completed studies (see our submitted manuscript in Appendix T) in the double adenosine $A_1$ and $A_{2A}$ receptor KO mice we developed under this project, in which the the individual and combined roles of these receptors have been probed in a mouse model of levodopa-induced dyskinesia (LID) in PD. Because the model entails a unilateral lesion to dopaminergic nigrostriatal projection using the locally (intrastrially) applied neurotoxin 6-hydroxydopamine, an important determination was the extent of the lesion (assessed as the amount of striatal dopamine loss relative to the contralateral unlesioned striatum) in the presence or absene of either or both of these adenosine receptors. We report that using this toxin neither the single or double KOs altered the lesion. The demonstration that the receptors were not

playing a critical role in this form of dopaminergic neuron injury, allowed for the main interpretations of this experiment to focus on the role of the $A_1$ and $A_{2A}$ receptors in LID. Surprisingly, we found that the $A_1$ as well as the $A_{2A}$ receptor was required for the full development of LID in this mouse model of PD. This finding prompted to assess the effect of caffeine, a non-specific adenosine (mixed $A_1$-$A_{2A}$) receptor antagonist, and we found that like the $A_1$-$A_{2A}$ double KO, the mixed $A_1$-$A_{2A}$ antagonist also reduced LID. The result suggests that early adjunctive use of adenosine antagonists (including caffeine) with levodopa, could help prevent the development of disabling LID in patients with PD.

- Also in Year 5 we have further investigated and preliminarily reported how depletion of adenosine $A_{2A}$ receptors (in $A_{2A}$ knockout mice) may mimic the neuroprotective effect of caffeine in another chronic (genetic) mouse model of PD. Here we used a lifelong exposure to an 'endogenous toxin' of transgenically expressed mutant human α–synuclein (*hw-αSYN*). Mutations in the human α–synuclein gene (A53T and A30P) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human α–synuclein gene with both of these mutations have been shown to develop age-related reductions in dopaminergic nigral neuron numbers, striatal dopamine (DA) and motor activity.

  We investigated whether deletion of the $A_{2A}$ receptor (in $A_{2A}$[-/-] mice) would afford protection against the age-related spontaneous reduction of striatal dopamine in the double-mutant human α -synuclein (*hm²-αSYN*) mice. Heterozygous $A_{2A}$[+/-] mice were mated with $A_{2A}$[+/-] mice that were also transgenic for the wild-type (*hw-αSYN*) or *hm²-αSYN* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter – all in a congenic C57Bl/6 background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional $A_{2A}$ receptor, DA content was significantly lower (by 35%) in hm²-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in $A_{2A}$[-/-] mice (lacking functional $A_{2A}$ receptor) the hm²-αSYN transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender.

  In Yr 5, we completed stereological nigral TH-IR cell counts clearly corroborating/confirming our observations with DA content above. Thus the $A_{2A}$ receptor appears to be required for neurotoxicity in an α–synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific $A_{2A}$ antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

***Specific Aim M2:*** to determine whether focal depletion of adenosine $A_{2A}$ receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity.

**Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed $A_{2A}$R mice**: Homozygous floxed $A_{2A}$R mice that previously received a stereotactic infusion of AAV-Cre or AAV-

green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP...

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Activity in Year 5 on SA M2 has primarily been in the expansion of our line of *floxed* $A_{2A}$ mice which is necessary to conduct the virally mediated focal eliminations of $A_{2A}$ receptors as planned. A period of reduced fertility/low offspring yields slowed the expansion. However after increasing the numbers of breeding pairs, the colony size has increased substantially in late 2009, with virally targeted Cre/*loxP* cKOs of $A_{2A}$ receptors in different brain regions now scheduled for January 2010. Other activity on the project in Year 5 has been the adoption in our laboratory of several measures of oxidative damage (nitrotyrosine and 4-hydroxy-2-nonenal [HNE], measure by immunohistochemistry or Western blotting) to be assessed in parallel with measures of dopanergic neuron injury and loss.

Also in Year 5 with this support of this award our laboratory has demonstrated that CSF levels of urate – a major antioxidant as well as the end product of adenosine metabolism in humans – along with those in serum are predictive clinical progression in patients with typical early PD, with publication of the work (Appendix Q) seen as a major advance in PD research. These findings have helped accelerate development of a clinical trial (http://clinicaltrials.gov/ct2/show/NCT00833690) of inosine (deamination product of adenosine and precursor of urate) to test its safety as a means to elevate urate in PD (http://www.michaeljfox.org/newsEvents_mjffInTheNews_pressReleases_article.cfm?ID=244).

## Body of the Report: for Yr 6

Progress during Year 6 on Specific Aims and experiments as laid out in our approved modification of the Statement of Work (SOW [in blue]) is described here in detail.

### STATEMENT OF WORK (focus on main areas of progress in Yr 6)

***Specific Aim #1*** – to definitively determine whether brain $A_{2A}$Rs or $A_1$Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain $A_{2A}$R is required for caffeine's protective effect in these PD models.

[and]

***Specific Aim M1:*** to definitively determine whether brain $A_{2A}$Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD.

- In this final year we completed and published our findings that caffeine protective effects extended to dopaminergic neuron degeneration in a chronic dual pesticide (paraquat plus maneb biweekly for months). See Appendix X.
- Similarly we were able to complete assessment and publication of the effects of $A_1$ KO, $A_{2A}$ KO and double $A_1$-$A_{2A}$ KO receptor knockout mouse (developed through this project) as well as caffeine in a related 6-OHDA toxin model of PD with superimposed levodopa-induced dyskinesias. See Appendix T.

- We also completed and published our characterization of the temporal relationship between exposures to caffeine and MPTP required for neuropotection by the former against toxicity of the latter. See Appendix W. In the same study we reported that natural caffeine metabilites (e.g., theophylline) that are also adenosine receptor antagonists are also neuroprotective in this standard toxin model of PD.

***Specific Aim #2*** – to localize the region within brain through which caffeine or $A_{2A}$ receptor inactivation produces its neuroprotective effect in the MPTP model of PD.

[and]

***Specific Aim M2:*** to determine whether focal depletion of adenosine $A_{2A}$ receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity.

**Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed $A_{2A}$R mice**: Homozygous floxed $A_{2A}$R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP...

- Continued progress was made in Year 6 toward the conditional KO-based localization of $A_{2A}$ receptors required for neruodegeneration with several dozen additional unilateral viral infusions into the striatum of 'floxed' $A_{2A}$ gene mice treated 6-8 weeks later with saline or MPTP. The processing and analyses of their brain tissues is currently under way.

***Specific Aim #3*** – to investigate caffeine-estrogen interactions in the MPTP model of PD by determining the effect estrogen replacement on the neuroprotective phenotype of A2A KO mice, and exploring potential peripheral and CNS mechanisms contributing to caffeine's reduced neuroprotective efficacy in the presence of estrogen.

- Having previously completed our caffeine-estrogen interaction studies in the MPTP model of PD, we were able to pursue related preliminary studies of potential sex differences in the effects of urate exposure caused by *urate oxidase* KO in both MPTP and 6-OHDA models of PD. The results of these studies are currently being analyzed. They build on human association data reported in Year 5 of this project and are of relevance to our recently initiated human studies of both women and men in a randomized control trial of inosine to elevate urate in early PD.
- We also characterized a neurotoxic (excitotoxic) influence within the local neuronal environment in a PD model, with the demonstration that metabotropic mGlu5 glutamate receptor KO mice are partially protected from the 6-OHDA toxicity. See Appendix V.

## **Key Research Accomplishments** (all Years)

### **Year 1** (from Yr 1 report)

- We have systematically demonstrated that estrogen can prevent caffeine's neuroprotective effect against dopaminergic neuron injury in the MPTP mosue model of Parkinson's disease (PD).
- In so doing, we have provided a biological basis for the consistent epidemiological finding that caffeine is associated with a reduced risk of PD in men and in women who have not had estrogen replacement therapy, but not in women who have had estrogen replacement therapy.
- We have demonstrated for the first time the use of virally delivered *cre* gene (via an AAV1-*cre* vector) that can discreetly eliminate the $A_{2A}$ receptor in brain. This powerful Cre-*loxP* methodology will allow us to dissect -- with exceptional molecular and anatomical precision -- the role of the adenosine $A_{2A}$ receptor in caffeine's influence on dopaminergic neuron injury in neurotoxin models of PD.
- We have established and expanded a novel double $A_1$-$A_{2A}$ double receptor knockout (KO) in preparation for experiments that can gauge the relative roles of the two major brain adenosine receptors in toxin models of PD.

### **Year 2** (from Yr 2 report)

- Confirmation of adenosine $A_{2A}$ receptor requirement for caffeine's neuroprotective effect in the MPTP model of neurodegeneration in Parkinson's disease.
- Demonstration that these receptors on forebrain neurons or astrocytes do not fully account for caffeine's neuroprotective action against MPTP toxicity, suggesting surprisingly that other neurons or glial cells are the source of the $A_{2A}$ receptors through which caffeine is acting.
- Characterization of a powerful Cre-*loxP* conditional knockout methodology that will allow us to dissect -- with exceptional molecular and anatomical precision -- the role of the adenosine $A_{2A}$ receptor in caffeine's influence on dopaminergic neuron injury in neurotoxin models of PD
- Publication of our systematic demonstration that estrogen can prevent caffeine's neuroprotective effect against dopaminergic neuron injury in the MPTP mosue model of Parkinson's disease (PD). In so doing, we have provided a biological basis for the consistent epidemiological finding that caffeine is associated with a reduced risk of PD in men and in women who have not had estrogen replacement therapy, but not in women who have had estrogen replacement therapy.

### **Year 3** (from Yr 3 report)

- Demonstration that neuronal forebrain $A_{2A}$ receptors can play a critical role in dopaminergic neuron injury in the MPTP model of neurodegeneration in Parkinson's disease.

- Using a powerful newly Cre/LoxP conditional knockout system, we have obtained evidence that it is the neuronal forebrain $A_{2A}$ receptors in the striatum that are responsible for this toxicity. Thus it is through these receptors that caffeine and more specific antagonists of the adenosine $A_{2A}$ receptor may offer neuroprotection against the development or progression of PD.
- Demonstration for the first time that caffeine's neuroprotective effect extends to the dual pesticide – parequat plus maneb – model of PD, a chronic, potentially more environmentally relevant model of the disease.
- Demonstration for the first time that urate can be neuroprotective in an *in vivo* model of PD. This finding may have a particularly rapid translational impact as urate-elevating therapy is now being pursued as potential neuroprotectant for PD patients.
- Methodological advances were achieved with a viral vector-based Cre/*LoxP* conditional knockout system. It will allow us to dissect caffeine and $A_{2A}$ receptor involvement in neurotoxin models of PD with an unprecedented combination of anatomical and molecular precision in different brain structures.

**Year 4** (from Yr 4 report)

- Completion of work and submission of a report of our demonstration that the non-specific adenosine antagonist caffeine can in fact confer neuroprotection in a chonic dual pesticide toxin (parequat plus maneb) model of PD, of potentially greater environmental relevance than prior toxin models of PD.
- Demonstration and communication of a protective effect of $A_{2A}$ receptor depletion on chronic dopaminergic neuron toxicity induced by transgenic expression of mutant human alpha-synuclein.
- Using a powerful newly Cre/LoxP conditional knockout system, we have preliminarily reported evidence that neuronal forebrain $A_{2A}$ receptors in the striatum contribute to this toxicity. Thus it is through these receptors that caffeine and more specific antagonists of the adenosine $A_{2A}$ receptor may offer neuroprotection against the development or progression of PD.

**Year 5** (from Yr 5 report)

- Achievement of key components of Aim M1, in demonstrating/publishing that targeted depletion of forebrain $A_{2A}$ receptors reduces dopaminergic neuron degeneration in a sub-chronic toxin model of PD. The findings strengthen the translational rationale for conducting clinical trials with adenosine $A_{2A}$ antagonists (either caffeine or more specific blockers) as a potential neuroprotective strategy.
- Identification and publication that higher CSF levels of the adenosine end product urate as predictor of favorable outcomes in PD. The findings are being rapidly translated into a major new clinical trial targeting urate elevation as a novel neuroprotective strategy in PD.
- Completion of preparations to conduct the key experiments under Aim M2 in which virally delivered cre gene will produce a focal conditional knockout of $A_{2A}$

receptors to further localize the mechanism by which $A_{2A}$ antagonists can confer neuroprotection in PD models.

### Year 6

- The discoveries that caffeine metabolites as well as caffeine are neuroprotective, and that caffeine can precede or follow toxin administration, enhances the plausibility that typical human exposures to caffeine could have a prolonged protective influence.
- In addition to its well established protective effect in multiple acute intoxication models of PD, caffeine was found to confer protection in a chronic dual pesitcide exposure model of PD. The study showed that dopaminergic neurons themselves were protected and preserved with regular exposure of a moderate dose of caffeine.
- Adenosine $A_1$ receptors as well as $A_{2A}$ receptors were shown to be required for the full development of abnormal dyskinetic responses to repeated levodopa administration in a toxin-based model of advanced PD. The findings demonstrate the utility of the dual knockout methodology and have therapeutic implications for novel strategies to avoid the development of dyskinetic motor complications of standard levodopa therapy in PD.
- Progress was made in establishing genetic methods to manipulate purine pathways in mice. We further validated a viral *cre*/*loxP* system for selectively disruption the *$A_{2A}$ receptor* gene in brain, and characterized the neurochemical phenotype of *urate oxidase* gene modifications. These tools will facilitate a better understanding of purine mechanisms and benefits in mouse models of PD.

## Reportable Outcomes

**Publications with acknowledgements citing W81XWH-04-1-0881/USAMRAA; see Appendices**

Abstracts

- Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease. Program No. 665.12. 2005 Washington, DC: Society for Neuroscience. [Abstract; **Appendix A**]
- Pisanu A, Sena-Esteves M, Schwarzschild MA. (2006) AAV-Cre/*loxP* conditional KO of adenosine $A_{2A}$ receptors in striatal neurons. *Targeting Adenosine $A_{2A}$ Receptors in Parkinson's Disease and Other CNS Disorders*. Boston May 17-19, 2006 www.A2APD.org P12. [Abstract; **Appendix B**]
- Pisanu A, Sena-Esteves M, Schwarzschild MA. (2006) Conditional knock-out of striatal adenosine $A_{2A}$ receptors using an adeno-associated virus-Cre/*loxP* system. Society for Neuroscience annual meeting, Atlanta. 199.23. [Abstract; **Appendix C**]

- Xu K, Xu Y-H, Chen J-F, Schwarzschild MA. (2006) The neuroprotection by caffeine in the MPTP model of Parkinson's disease is lost in adenosine $A_{2A}$ knockout mice. *Targeting Adenosine $A_{2A}$ Receptors in Parkinson's Disease and Other CNS Disorders*. Boston May 17-19, 2006 www.A2APD.org P30. [Abstract; **Appendix D**]

- Xu K, Yu Y-H, Chen J-F, Schwarzschild MA. (2006) Neuroprotection by caffeine in the MPTP model of Parkinson's disease: the role of adenosine $A_{2A}$ receptor. Society for Neuroscience annual meeting, Atlanta. 470.17. [Abstract; **Appendix E**]

- Kachroo A, Prasad K, Irizarry MC, Richfield EK, Schwarzschild MA. 2007. Caffeine protects against combined paraquat and maneb-induced neurotoxicity of dopaminergic nigral neurons. 2007 Neuroscience Meeting. San Diego, CA: Society for Neuroscience. Abstract # 265.20/U10. . [Abstract; **Appendix F**]

- Pisanu A, Sena-Esteves M, Schwarzschild MA. 2007. Behavioural phenotype of unilateral conditional knockout of striatal adenosine A2A receptors using a viral Cre/loxP system. 2007 Neuroscience Meeting. San Diego, CA: Society for Neuroscience. Abstract # 314.7/GGG27. [Abstract; **Appendix G**]

- Luo W, Schwarzschild MA, Xu K. 2007. Urate attenuates MPTP-induced dopaminergic neurotoxicity in mice. 2007 Neuroscience Meeting. San Diego, CA: Society for Neuroscience. Abstract # 797.8/O12. [Abstract; **Appendix H**]

- Kachroo A, Richfield EK, Schwarzschild MA. 2008. Adenosine A2A receptor gene deletion confers protection in a transgenic alpha-synuclein model of Parkinson's disease. 2008 Neuroscience Meeting. Washington, DC: Society for Neuroscience. Abstract # 453.14/CC8. [Abstract; **Appendix I**]

- Pisanu A, Sena-Esteves M, Schwarzschild MA. 2008. Effect of conditional knockout of striatal adenosine A2A receptors on MPTP-induced nigrostriatal toxicity in mice. 2008 Neuroscience Meeting. Washington, DC: Society for Neuroscience. Abstract # 453.6/BB34. [Abstract; **Appendix J**]

- Kachroo A, Richfield EK, Schwarzschild MA. 2009. Adenosine A2A receptor gene deletion confers protection of nigral neurons in an alpha-synuclein model of Parkinson's disease. 2008 Neuroscience Meeting. Chicago, IL: Society for Neuroscience. Abstract # 142.5. [Abstract; **Appendix K**]

- Schwarzschild MA. 2010. Purines and diseases of the basal ganglia. 10th Meeting of the International Basal Ganglia Society. IBAGS X. Long Branch, NJ: USA. Abstract # O-43; pg 48. [Abstract; **Appendix L**]

Journal Publications

- Hauser R, Schwarzschild MA. 2005. Adenosine A2A receptors for Parkinson's disease. *Drugs and Aging* 22:471-482. [**Appendix M**]

- Xu K, Xu Y, Brown-Jermyn D, Chen JF, Ascherio A, Dluzen DE, Schwarzschild MA. (2006) Estrogen prevents neuroprotection by caffeine in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *J Neurosci*. 26:535-541. [**Appendix N**]

- Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M. (2006) Targeting adenosine $A_{2A}$ receptors in Parkinson's disease. *Trends Neurosci*. 29:647-654. [**Appendix O**]

- Hung AY, Schwarzschild MA. 2007. Clinical trials for neuroprotection in Parkinson's disease: overcoming angst and futility? *Curr Opin Neurol*. 20:477-483. [**Appendix P**]

- Ascherio A, LeWitt PA, Xu K, Eberly S, Watts A, Matson WR, Marras C, Kieburtz K, Rudolph A, Bogdanov MB, Schwid SR, Tennis M, Tanner CM, Beal MF, Lang AE, Oakes D, Fahn S, Shoulson I, Schwarzschild MA; Parkinson Study Group DATATOP Investigators. (2009) Urate as a predictor of the rate of clinical decline in Parkinson disease. *Arch Neurol*. 66:1460-1468. [**Appendix Q**]

- Carta AR, Kachroo A, Schintu N, Xu K, Schwarzschild MA, Wardas J, Morelli M. (2009) Inactivation of neuronal forebrain $A_{2A}$ receptors protects dopaminergic neurons in a mouse model of Parkinson's disease. *J Neurochem*. 111:1478-1489. [**Appendix R**]

- McFarland NR, Fan Z, Xu K, Schwarzschild MA, Feany MB, Hyman BT, McLean PJ. (2009) Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease. *J Neuropathol Exp Neurol*. 68:515-524. [**Appendix S**]

- Xiao D, Cassin JJ, Healy B, Burdett TC, Chen JF, Fredholm BB, Schwarzschild MA. (2010) Deletion of adenosine $A_1$ or $A_{2A}$ receptors reduces L-3,4-dihydroxyphenylalanine-induced dyskinesia in a model of Parkinson's disease. *Brain Res*. Sep 6, 2010 [Epub ahead of print] [**Appendix T**]

- Morelli M, Carta AR, Kachroo A, Schwarzschild MA. (2010) Pathophysiological roles for purines: adenosine, caffeine and urate. *Prog Brain Res*. 183:183-208. [**Appendix U**]

- Black YD, Xiao D, Pellegrino D, Kachroo A, Brownell AL, Schwarzschild MA. (2010) Protective effect of metabotropic glutamate mGluR5 receptor elimination in a 6-hydroxydopamine model of Parkinson's disease. *Neurosci Lett*. Sep 18, 2010. [Epub ahead of print] [**Appendix V**]

- Xu K, Xu YH, Chen JF, Schwarzschild MA. (2010) Neuroprotection by caffeine: time course and role of its metabolites in the MPTP model of Parkinson's disease. *Neuroscience*. 167:475-481. [**Appendix W**]

- Kachroo A, Irizarry MC, Schwarzschild MA. (2010) Caffeine protects against combined paraquat and maneb-induced dopaminergic neuron degeneration. *Exp Neurol*. 223:657-661. [**Appendix X**]

**Presentations (with acknowledgements made to DoD/USAMRAA/NETRP)**

- April 6, 2005 – (Austin) University of Texas, SW – grand rounds, "*Caffeine, adenosine receptors and Parkinson's disease*""
- September 7, 2005 – Polish Neuroscience Society; 9/7/05 symposium speaker, "*Caffeine, adenosine $A_{2A}$ receptors and neuroprotection in PD*"
- September 16, 2005 (New York City) – Michael J. Fox Foundation for Parkinson's Research, "*Dyskinesia Summit*"
- September 26, 2005 (Philadelphia) Univ. of Penn – Dept of Pharmacology seminar, "*Caffeine, adenosine $A_{2A}$ receptors and Parkinson's disease*"
- January 4, 2006 – (Piscatway) University of New Jersey Medicine & Dentistry – grand rounds, "*Caffeine, adenosine $A_{2A}$ receptors and Parkinson's disease*"
- February 25, 2006 –World Parkinson Congress – Washington, DC "*Neuroprotective and Anti-dyskinetic Potential of Adenosine $A_{2A}$ Blockers*"
- March 27, 2006 –American Chemical Society – Atlanta [Co-chair] Symposium: *Current and Future Potential Drug Therapy for Parkinson's Disease*; "*Progress on the etiology, modeling and treatment of Parkinson's disease*"
- May 18, 2006 – *Targeting Adenosine $A_{2A}$ Receptors in Parkinson's Disease and Other CNS Disorders*. (Organizer/Co-chair) Boston May 17-19, "*$A_{2A}$ in L-dopa sensitization/dyskinesia models*".
- October 18, 2006 – Society for Neuroscience – Atlanta (Symposium: Purinergic Signaling in Neuron-Glia Interactions) "*Adenosine $A_{2A}$ Receptors in Neurodegeneration*" {Eweson Lectureship}
- April 5, 2007, 5$^{th}$ Annual NIEHS/CCPDER Meeting (Asilomar/Monterey) "Purines & Parkinson's: Pursuing environmental exposures linked to reduced risk" {keynote speaker}.
- June 27, 2007, Parkinson's Disease and Environment Consensus Conference (Sunnyvale, CA) "Metabolic Concerns: Serum urate predicts progression of Parkinson's disease" {panel presenter}.
- July 15, 2007 – International Brain Research Organization (IBRO) World Congress of Neuroscience. (Melbourne) Symposium: "*Role of adenosine $A_{2A}$ receptors in noxious brain conditions: effects on neurons, astrocytes or microglia?*" {chair; presenter}.
- 2008 – "PD & Purines: from adenosine to urate" (Biogen-Idec Lecture Series; Cambridge, MA), {Invited Lecture}

- 2008 – "Pursuing Parkinson's with Purines" (Univ. of Otawa), University of Otawa, {Invited Lecture}
- 2008 – Antixodant & Antiinflammatory Strategies in Parkinson's Disease (Royal Society of Medicine Symposium, London), Cure Parkinson's Trust {Invited Lecture}
- 2008 – "Purinergic Therapeutics for Parkinson's Disease"; Soochow Univ., Suzhou, China, {Invited Lecture}
- 2009 –"Purine Targets in Neurotherapeutics: Adenosine, caffeine & urate in Parkinson's" (Winter Conference on Brain Research; Copper Mtn, CO) , American Federation for Aging Research{ Invited Eweson Lectureship program.}
- 2009 – UPenn Neurology Grand Rounds: "Pursuing Purines in Parkinson's: from caffeine to adenosine to urate", Univ Penn - Neurology Dept.
- 2010 – "Purine Pieces to the Parkinson's Puzzle" (Mt. Sinai Medical School, NY), Mt. Sinai Medical School,, {Invited Lecture}
- 2010 – "Targeting Purines in Parkinsonís: From Adenosine to Caffeine to Urate" (Tarragona, Spain), Purines 2010
  ntation}
- 2010 – IBAGS X -- "Purines & Basal Ganglia Diseases: Targeting adenosine, caffeine & urate" (Long Beach, NJ USA), International Basal Ganglia Society.
- 2010 – "Pursuing Purine Therapies in Parkinsonís" (Capital Medical University; Beijing, China) , Capital Medical University; Xuanwu Hospital. {Invited Lecture}
- 2010 – Soochow University Lecture "Therapeutic Neuroprotectants for Parkinsonís Disease" (Souzhou, China), Neurology Dept; 2nd Affiliated Hospital of Soochow University Lecture {Invited Lecture}
- 2010 – Faculdade de Medicina de Lisboa Lecture "Urate in Parkinsonís: from Apes to Antioxidant Trials" (Lisbon, Portugal), Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa.
- 2010 – World Parkinson Congress "Caffeine, Adenosine & Urate: from Molecular Epi to Drug Trials " (Glasgow, Scotland/UK), World Parkinson Congress/

## Conclusions/Plans

Our progress under this award has strengthened the central hypothesis that *Multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease*. In particular, we have explored the interactions between purines (primarily caffeine and adenosine, and more recently urate) with estrogen and environmentally relevant toxins in mouse models of PD.

Mechanistic questions remain over the role of purines in PD (e.g., as to how caffeine acts on adenosine $A_{2A}$ receptors to protect neurons) and will be the focus of continued investigation. The results have had a discernable impact on our understanding of PD pathophysiology and epidemiology, and have helped accelerate clinical applications of these biological insights. Specifically,

- Our demonstration that caffeine is consistently neuroprotective in both acute toxin and pesticide models of PD have strengthened the evidence that environmental factors like caffeine may offer realistic approaches to avoiding or reducing the risk of PD.
- The discovery that caffeine metabolites (theophylline and paraxanthine) as well as caffeine are neuroprotective enhances the plausibility that typical human exposures to caffeine could have a prolonged protective influence sufficient to account for the inverse epidemiological link between coffee or caffeine consumption and PD risk.
- With our substantiation of adenosine $A_{2A}$ antagonism as the basis for putative neuroprtection by caffeine, we have strengthened the evidence that caffeine and other adenosine $A_{2A}$ antagonists have potential disease-modifying benefits in PD patients. Of note the project findings support the first disease-modiciation trial design (large [~1000 subject] early PD cohort; 'delayed-start'; rasagiline comparator; long-term [1 yr] follow-up) for an adenosine $A_{2A}$ antagonist. (See http://clinicaltrials.gov/ct2/show/NCT01155479 .)
- The extension of the inverse caffeine-PD link to a similarly compelling inverse link between urate – a related purine and antioxidant – and PD progression as well as risk has major translational significance. It has raised multiple novel mechanistic hypothesis now being pursued and has also prompted rapid clinical translation to a trial of urate elevation in PD. See http://clinicaltrials.gov/ct2/show/NCT00833690 .)

**Complete List of all Personnel** (all MGH employees '04-10) paid from this award:

- Brown-Jermyn, Deborah
- Chen, Melissa
- Chen, Xiqun
- Cipriani, Sara
- Kachroo, Anil
- Mohsin, Mujaddid
- Pisanu, Augusta
- Schwarzschild, Michael
- Xiao, Danqing
- Xu, Kui
- Xu, Yuehang

**Program Number:** 665.12 **Day / Time:** Tuesday, Nov. 15, 11:00 AM - 12:00 PM

# Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease

K.Xu[1*]; Y.Xu[1]; D.Brown-Jermyn[1]; J.F.Chen[2]; A.Ascherio[3]; D.E.Dluzen[4]; M.A.Schwarzschild[1]

*1. MGH-MIND, Charlestown, MA, USA; 2. BU, Boston, MA, USA; 3. HSPH, Boston, MA, USA; 4. NEOUCOM, Rootstown, OH, USA*

Epidemiological studies have linked caffeine consumption with a reduced risk of Parkinson's disease (PD) in men. Interestingly, in women this inverse association is present only in those who have not used postmenopausal estrogens, suggesting an interaction between the influences of estrogen and caffeine use on the risk of PD. To explore a possible biological basis for an estrogen-caffeine interaction in a mouse model of PD, we investigated the effect of caffeine on MPTP neurotoxicity, first in young or retired breeder (RB) male and female mice, then in ovariectomized (OVX) female mice or intact male mice implanted with estrogen or placebo pellets. Caffeine (0-40 mg/kg ip) was administered 10 min prior to a single MPTP injection (40 mg/kg ip). One week later striatal dopamine content was determined. 1. Caffeine treatment produced a dose-dependent attenuation of MPTP-induced striatal dopamine loss in both young and RB male mice. However, in female mice, only the highest dose of caffeine provided similar protection. 2. Lower doses of caffeine significantly reduced dopamine loss in OVX or OVX+placebo mice, but not in sham-operated or OVX + estrogen young female mice. Moreover, in OVX RB female mice, caffeine treatment, both lower and higher doses, significantly attenuated MPTP-induced dopamine loss only in placebo, but not in estrogen treated mice. 3. Caffeine's protection against dopamine depletion in male mice was lost after estrogen treatment. 4. There is no overall difference in brain levels of caffeine and its metabolites between OVX+placebo and OVX+estrogen mice. Taken together, these results demonstrate that estrogen reduces caffeine's neuroprotective effect in both male and female mice in the MPTP model of PD. They suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD, and provide a model for exploring further the mechanism of this interaction.

*Support Contributed By: NIEHS,Beeson Program/AFAR & USAMRAA.*

**Citation:** K.Xu, Y.Xu, D.Brown-Jermyn, J.F.Chen, A.Ascherio, D.E.Dluzen, M.A.Schwarzschild. Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease. Program No. 665.12. *2005 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience

**TARGETING ADENOSINE $A_{2A}$ RECEPTORS IN PARKINSON'S DISEASE AND OTHER CNS DISORDERS**

May 17 - 19, 2006
Boston USA

*presented by*
MassGeneral Institute for Neurodegenerative Disease

# AAV-Cre/*loxP* conditional KO of adenosine $A_{2A}$ receptors in striatal neurons

Augusta Pisanu*, Miguel Sena-Esteves & Michael A. Schwarzschild

*Department of Neurology, Massachusetts General Hospital, Charlestown, MA USA;*
*Presenting author (apisanu@partners.org.)

Gene knockout (KO) approaches to receptor function complement traditional pharmacological methods by complete specificity and inactivation. However, standard KO strategies globally eliminate the targeted receptor, and thus their use for investigating the role of receptors in the adult brain can be confounded by developmental or systemic phenotypes. The transgenic Cre/*loxP* conditional KO system can achieve partial control over the timing and distribution of receptor inactivation using a specific promoter to direct *cre* expression, e.g., to study the effects of the adenosine $A_{2A}$ receptor ($A_{2A}R$) in postnatal forebrain. To achieve an even higher degree of precision in eliminating the $A_{2A}R$ from discrete regions on one side of the brain, and in pursuit of a better understanding of $A_{2A}R$ involvement in neurodegeneration, we adopted an AAV-Cre/*loxP* system. Adeno-associated virus (AAV)-Cre vectors were stereotactically infused into homozygous *floxed $A_{2A}R$* adult mice, resulting in a conditional KO of the $A_{2A}R$ at the site and time of infection.

Working with several serotypes of AAV-Cre and AAV-GFP, injected into the striatum of *floxed $A_{2A}R$* mice, we demonstrated an infection and GFP expression largely restricted to the targeted striatum for AAV1 serotype (Fig. A) but not AAV1/8 (which produced a widespread infection extending into the overlying cortex). Focusing on AAV1 serotype we characterized the titer-dependence and time-course of neuronal Cre expression and $A_{2A}R$ loss, visualized by IHC (as in Fig. B). Cre expression was detectable 8 days post-infusion of AAV vectors but the loss of $A_{2A}R$ was not evident until the 16$^{th}$ day, reaching a maximum extent at the 32$^{nd}$ day post-injection.

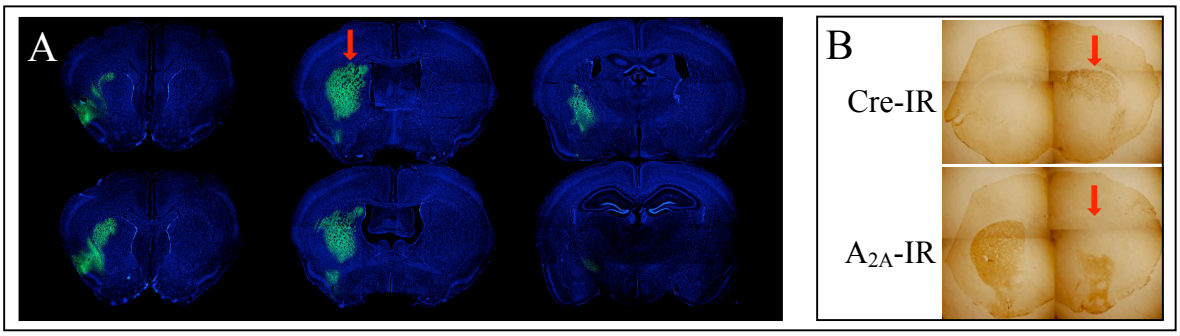


Figure: (**A**) Fluorescence of rostral→caudal coronal sections stained with a Hoechst 33258 one month after AAV1-GFP infusion (arrow). (**B**) Striatal Cre expression and the coincident elimination of striatal $A_{2A}R$ in a *floxed $A_{2A}R$* mouse one month after unilateral intrastriatal injection of AAV1-Cre (arrow).

The AAV-Cre/*loxP* conditional KO system provides a precise tool with which to explore the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

*Funded by DoD W81XWH-04-1-0881.*

To the best of my knowledge and judgment I, the presenting author, report that:
[Please check one.]

☑ The authors have no financial conflict of interest‡ in the presentation of this work.

☐ The authors may have a financial conflict(s) of interest‡ as I have described on the next page.

‡ Significant *financial conflict of interest* is explained under the abstract submission policy (rule #11) and scientific communication guildelines (section 1.6) of the Society for Neuroscience.

Appendix F

Appendix C



**Program#/Poster#:** 199.23/OO90

**Title:** Conditional knock-out of striatal adenosine A2A receptors using an adeno-associated virus-Cre/loxP system

**Location:** Georgia World Congress Center: Halls B3-B5

**Presentation Start/End Time:** Sunday, Oct 15, 2006, 10:00 AM -11:00 AM

**Authors:** ***A. PISANU**, M. SENA-ESTEVES, M. A. SCHWARZSCHILD;
Neurology, Massachusetts General Hospital, Charlestown, MA.

Gene knockout (KO) approaches to the study of receptor function complement traditional pharmacological methods by providing complete inactivation and specificity. However, standard KO strategies globally eliminate the targeted receptor starting prenatally, and thus their use for investigating the role of receptors in the adult brain can be confounded by developmental or systemic phenotypes. The transgenic Cre/*loxP* conditional KO (cKO) system can achieve partial control over the timing and distribution of receptor inactivation using a specific promoter to direct Cre recombinase expression postnatally and/or in a selected cell subtype [e.g. *CaMK-IIα* driven-*cre* to study the effects of the adenosine $A_{2A}$ receptor ($A_{2A}R$) in postnatal forebrain].

To achieve an even higher degree of precision in eliminating the $A_{2A}R$ from discrete regions on one side of the brain, and in pursuit of a better understanding of $A_{2A}R$ involvement in neurodegeneration, we adopted an AAV-Cre/*loxP* system. Adeno-associated virus (AAV)-Cre vectors were stereotactically infused (with an injection volume of 1 µl) into the left striatum of homozygous floxed $A_{2A}R$ adult mice, resulting in a cKO of the $A_{2A}R$ at the site and time of infection. The titer-dependence and time-course of neuronal Cre expression and $A_{2A}R$ loss, were characterized and visualized by immunohistochemistry. Cre expression was detectable 8 days post-infusion of AAV1 serotype vectors but the loss of $A_{2A}R$ was not evident until the $16^{th}$ day, reaching a maximum extent at the $32^{nd}$ day post-injection. The extent of the infected area depended on the titer of the infused vectors, estimated at 20% of calculated volume of the striatum using a low titer of $10^{12}$ gc/ml and reaching 80% when a higher titer of $10^{14}$ gc/ml was infused. In this model, the AAV-Cre/*loxP* cKO system provides a temporally and spatially controlled method to precisely eliminate $A_{2A}R$ in discrete brain regions providing additional advantages in exploring the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

**Disclosures:** **A. Pisanu** , None; **M. Sena-Esteves**, None; **M.A. Schwarzschild**, None.

**Support:** DoD W81XWH-04-1-0881

Appendix C

Appendix D

P-30


# Targeting Adenosine $A_{2A}$ Receptors in Parkinson's Disease and Other CNS Disorders

May 17 - 19, 2006
Boston USA

presented by
MassGeneral Institute for Neurodegenerative Disease

**The neuroprotection by caffeine in the MPTP model of Parkinson's disease is lost in adenosine $A_{2A}$ knockout mice.**

Kui Xu[1]*, Yue-Hang Xu[1], Jiang-Fan Chen[2], Michael A. Schwarzschild[1]

*1. MassGeneral Institute for Neurodegenerative Disease, Charlestown, MA, USA. 2. Boston University, Boston, MA, USA.* *Presenting author (xu@helix.mgh.harvard.edu)

**Background:** Prospective epidemiological studies have raised the possibility of caffeine conferring protection against Parkinson's disease. This hypothesis is strengthened by our previous findings that caffeine attenuates MPTP-induced dopaminergic neurotoxicity in mice. Moreover, antagonists of the $A_{2A}$ subtype of adenosine receptor ($A_{2A}R$), but not of the $A_1R$, provided similar protection. To further investigate the dependence upon and location of the $A_{2A}R$ in caffeine's neuroprotection, we examined the effect of caffeine on MPTP neurotoxicity in standard (global) $A_{2A}R$ knockout ($A_{2A}$ KO) mice as well as tissue-specific (conditional) $A_{2A}$ KO mice.
**Methods:** Postnatal forebrain neuron-specific and astrocyte-directed conditional $A_{2A}$ KO mice were generated by using the Cre-*loxP* system based on the specificity of *CamKII*$\alpha$ and *GFAP* gene promoters, respectively. Tissue-specific disruption of the $A_{2A}R$ was confirmed by PCR and western blot. Locomotion, scored as the number of adjacent photobeam breaks (*Ambulation*), was determined 3 hr before and 3 hr after caffeine/saline injection in wide-type and knockout mice. In neuroprotection experiment, caffeine or saline were administered 10 minutes before MPTP treatment (40 mg/kg ip single injection). One week later, striatal dopamine content was determined by HPLC.
**Results:** Caffeine-stimulated locomotion is significantly decreased in forebrain neuron-specific $A_{2A}R$ KO mice, similar to what we found previously in global $A_{2A}R$ KO mice. MPTP treatment (40 mg/kg single injection) produced similar dopamine depletion in knockout mice and their respective wide-type littermates. On the other hand, caffeine pretreatment (25 mg/kg ip) significantly attenuated MPTP-induced striatal dopamine loss in wild-type mice. This neuroprotection by caffeine, however, is lost in global $A_{2A}R$ KO mice. Similarly, caffeine attenuated MPTP-induced dopamine depletion in control but not forebrain neuron-specific $A_{2A}R$ KO mice. On the other hand, caffeine's attenuation of MPTP neurotoxicity is present in both control and astrocyte-directed $A_{2A}R$ KO mice.
**Conclusions:** Taken together, these data suggest that caffeine's neuroprotection against MPTP neurotoxicity is dependent on the $A_{2A}R$, particularly those located in forebrain neurons.

*Support contributed by: NIEHS, Beeson Program/AFAR & USAMRAA.*

To the best of my knowledge and judgment I, the presenting author, report that:
The authors have no financial conflict of interest in the presentation of this work.

Appendix E

Appendix E



**Program#/Poster#:** 470.17/LL43

**Title:** Neuroprotection by caffeine in the MPTP model of Parkinson's disease: the role of adenosine A2A receptor

**Location:** Georgia World Congress Center: Halls B3-B5

**Presentation Start/End Time:** Monday, Oct 16, 2006, 1:00 PM - 2:00 PM

**Authors:** ***K. XU**[1], Y. XU[1], J. CHEN[2], M. A. SCHWARZSCHILD[1];

[1]Dept Neurol, Massachusetts Gen Hsptl, Charlestown, MA, [2]Neurology, Boston University School of Medicine, Boston, MA.

Prospective epidemiological studies have raised the possibility of caffeine conferring protection against Parkinson's disease. This hypothesis is strengthened by our previous findings that caffeine attenuates MPTP-induced dopaminergic neurotoxicity in mice. Moreover, antagonists of the $A_{2A}$ subtype of adenosine receptor ($A_{2A}$R), but not of the $A_1$R, provided similar protection. To further investigate the dependence upon and location of the $A_{2A}$R in caffeine's neuroprotection, we examined the effect of caffeine on MPTP neurotoxicity in standard (global) $A_{2A}$R knockout ($A_{2A}$ KO) mice as well as tissue-specific (conditional) $A_{2A}$ KO mice. Postnatal forebrain neuron-specific and astrocyte-directed conditional $A_{2A}$ KO mice were generated by using the Cre-loxP system based on the specificity of CamKIIα and GFAP gene promoters, respectively. Tissue-specific disruption of the $A_{2A}$R was confirmed by PCR and western blot. Locomotion (ambulation) was determined 3 hr before and 3 hr after caffeine/saline injection. In neuroprotection experiment, caffeine or saline were administered 10 minutes before MPTP treatment (40 mg/kg ip single injection). One week later, striatal dopamine content was determined by HPLC. Caffeine's effect on MPTP-induced dopamine release will also be studied in the conditional $A_{2A}$ KO mice using microdialysis. Caffeine-stimulated locomotion is significantly decreased in forebrain neuron-specific $A_{2A}$R KO mice, similar to what we found previously in global $A_{2A}$R KO mice. MPTP treatment (using the single dose injection paradigm) produced similar dopamine depletion in global KO mice and their wide-type or heterozygous littermates. By contrast, caffeine pretreatment (25 mg/kg ip) significantly attenuated MPTP-induced striatal dopamine loss in wild-type and heterozygous mice but not in global $A_{2A}$R KO mice. Similarly, caffeine attenuated MPTP-induced dopamine depletion in control but not forebrain neuron-specific $A_{2A}$R KO mice. On the other hand, caffeine's attenuation of MPTP neurotoxicity is present in both control and astrocyte-directed $A_{2A}$R KO mice. Taken together, these data suggest that caffeine's neuroprotection against MPTP neurotoxicity is dependent on $A_{2A}$ receptors, particularly those located on forebrain neurons.

**Disclosures:** **K. Xu** , None; **Y. Xu**, None; **J. Chen**, None; **M.A. Schwarzschild**, None.

**Support:** NIH ES10804

Beeson Program/AFAr

USAMRAA W81XWH-04-1-0881

**Program#/Poster#:** 265.20/U10

**Title:** Caffeine protects against combined paraquat and maneb-induced neurotoxicity of dopaminergic nigral neurons

**Location:** San Diego Convention Center: Halls B-H

**Presentation Start/End Time:** Sunday, Nov 04, 2007, 3:00 PM - 4:00 PM

**Authors:** ***A. KACHROO**[1], K. PRASAD[2], M. C. IRIZARRY[1], E. K. RICHFIELD[2], M. A. SCHWARZSCHILD[1];
[1]Dept of Neurol., Mass Gen. Hosp/Harvard Med. Sch., Charlestown, MA;
[2]Envrn. and Occup. Hlth. Sci. Institute, Univ. of Med. and Dent. of New Jersey, Piscataway, NJ

The etiology of idiopathic Parkinson's disease (PD) remains unknown though certain risk factors have been identified. On the one hand, genetic determinants and environmental neurotoxicants such as pesticides have been linked to an increased risk of developing PD. On the other hand, several environmental factors such as caffeine intake have been inversely linked to PD risk. The present study sought to determine whether caffeine (a non-specific adenosine antagonist) attenuates dopamine neuron toxicity from combined exposure to the herbicide paraquat (PQ) and the fungicide maneb (MB). The intraperitoneal injection paradigm involved dual administration of 10mg/kg PQ and 30mg/kg MB, in the absence or presence of caffeine at 5mg/kg and 20mg/kg (injected 10 min prior to pesticides), twice a week for 8 weeks. PQ/MB treated mice did not demonstrate any motor deficits when tested 24-48 h post injection, using open field locomotor activity. Stereological assessment of neurons in the substantia nigra pars compacta was performed using tissue sections stained for tyrosine hydroxylase (TH) and counterstained with cresyl violet. The data showed that the numbers of TH-positive (dopaminergic) nigral neurons were significantly reduced (40%) after paraquat and maneb treatment. Pretreatment with caffeine at 20mg/kg, but not 5mg/kg, provided significant protection against TH-positive neuronal cell loss. The lack of a significant difference observed between control and pesticide-treated groups when counting TH-negative (non-dopaminergic) neurons confirms the specificity of these pesticides for targeting dopaminergic neurons. These data demonstrate that caffeine is able to neuroprotect dopaminergic nigral neurons in the setting of an environmentally relevant model of PD.

**Disclosures:** **A. Kachroo**, None; **K. Prasad**, None; **M.C. Irizarry**, None; **E.K. Richfield**, None; **M.A. Schwarzschild**, None.

**Support:** NIEHS Grant R01010804

DOD Grant W81XWH-04-1-0881

AFAR/Beeson program

[Authors]. [Abstract Title]. Program No. XXX.XX. 2007 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2007. Online.

**Program#/Poster#:** 314.7/GGG27

**Title:** Behavioural phenotype of unilateral conditional knockout of striatal adenosine A2A receptors using a viral Cre/loxP system

**Location:** San Diego Convention Center: Halls B-H

**Presentation Start/End Time:** Sunday, Nov 04, 2007, 3:00 PM - 4:00 PM

**Authors:** ***A. PISANU**, M. SENA-ESTEVES, M. A. SCHWARZSCHILD; Neurol., Massachusetts Gen. Hosp., Charlestown, MA

Genetic knockout (KO) approaches to the study of receptor function complement traditional pharmacological methods by offering complete specificity and inactivation. However, standard KO strategies globally eliminate the targeted receptor starting prenatally, and thus their use for investigating the role of receptors in the adult brain can be confounded by developmental or systemic phenotypes. The transgenic Cre/*loxP* conditional KO system can achieve partial control over the timing and distribution of receptor inactivation using a specific promoter to direct Cre recombinase gene (*cre*) expression postnatally and/or in a selected cell subtype. $A_{2A}$ receptors ($A_{2A}R$) have been reported to modulate motor functions in basal ganglia circuitry and their antagonists possess anti-parkinsonian activity in animal models. Convergent epidemiological and laboratory data have also suggested that $A_{2A}R$ blockade may confer neuroprotection against the dopaminergic neuron degeneration that causes Parkinson's Disease. Stereotaxic infusion of an adeno-associated virus (AAV) vector encoding Cre recombinase into the left striatum of homozygous floxed $A_{2A}R$ ($A_{2A}R^{flox/flox}$) adult mice was used to analyze the role of striatal $A_{2A}R$ in motor control and neuroprotection. Neuronal Cre expression and $A_{2A}R$ loss were visualized by immunohistochemistry and correlated with turning behaviour to assess for asymmetries in striatal control of movement. Cre expression appeared maximal 15 days post-infusion of AAV2/1-*cre* vector whereas loss of $A_{2A}R$ immunoreactivity reached its maximum extent at 30 days post-injection. Starting from the $30^{th}$ day turning behaviour was assessed in unilateral conditional KO mice (AAV2/1-*cre*, $A_{2A}R^{flox/flox}$) and in two control groups (AAV2/1-*GFP*, $A_{2A}R^{flox/flox}$ and AAV2/1-*cre*, $A_{2A}R^{+/+}$ mice). None of the experimental groups showed a biased spontaneous turning behaviour. In unilateral conditional KO mice the $A_{2A}$ antagonist KW-6002 (3 mg/kg, ip; in the presence of a sub-threshold dose of the dopamine $D_2$ antagonist haloperidol) induced ipsilateral turning, and amphetamine (2.5 mg/kg i.p. on its own) induced contralateral turning relative to that in the control animals. Thus the AAV2/1-Cre/*loxP* conditional KO system provides a means to precisely eliminate the $A_{2A}R$ postnatally in discrete brain regions, and can be used to further explore the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

**Disclosures:** **A. Pisanu** , None; **M. Sena-Esteves**, None; **M.A. Schwarzschild**, None.

**Support:** DoD W81XWH-04-1-0881

[Authors]. [Abstract Title]. Program No. XXX.XX. 2007 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2007. Online.

**Program#/Poster#:** 797.8/O12

**Title:** Urate attenuates MPTP-induced dopaminergic neurotoxicity in mice

**Location:** San Diego Convention Center: Halls B-H

**Presentation Start/End Time:** Wednesday, Nov 07, 2007, 11:00 AM -12:00 PM

**Authors:** **W. LUO**[1,2], M. A. SCHWARZSCHILD[2], *K. XU[2];
[1]Neurol., Second Affliated Hosp. Soochow Univ., Suzhou, China; [2]Dept Neurol, Massachusetts Gen Hsptl, Charlestown, MA

Findings from large prospectively followed populations have firmly linked higher urate level with a reduced risk of developing Parkinson's disease (PD). Recently, higher serum urate has been identified as a predictor of slower progression of PD symptoms and signs in two long-term, rigorously conducted clinical trials (together comprising over 1600 early cases of PD). These observations raise the possibility of a novel neuroprotective effect of urate in PD, and prompted us to explore its role in an animal model of PD. We examined the effect of locally administered urate on MPTP toxicity in young male C57Bl/6 mice. Urate (0, 2.5, 5, 7.5, or 10 mg/dL in artificial CSF) was perfused through microdialysis probe within a guide canula into the left lateral ventricle of free moving mice for 48 hours. MPTP-HCl (single ip injection of 40 mg/kg) or saline was given 24 hours after the start of urate perfusion. One week after MPTP, striatal dopamine content was determined. Urate (5mg/dL but not at higher dose) perfusion significantly attenuated MPTP-induced striatal dopamine loss. These preliminary data support a neuroprotective role of urate in the MPTP mouse model of PD.

**Disclosures:** **W. Luo**, None; **M.A. Schwarzschild**, None; **K. Xu** , None.

**Support:** Jiangsu Government Scholarship for Oversea Studies(JS-2003-106), China
USAMRAA W81XWH-04-1-0881

[Authors]. [Abstract Title]. Program No. XXX.XX. 2007 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2007. Online.

Appendix I

Print this Page


Presentation Abstract

| | |
|---|---|
| Program#/Poster#: | 453.14/CC8 |
| Title: | Adenosine $A_{2A}$ receptor gene deletion confers protection in a transgenic α-synuclein model of Parkinson's disease |
| Location: | Washington Convention Center: Hall A-C |
| Presentation Time: | Monday, Nov 17, 2008, 2:00 PM - 3:00 PM |
| Authors: | ***A. KACHROO**[1], E. K. RICHFIELD[2], M. A. SCHWARZSCHILD[1]; [1]Dept of Neurol., Mass Gen. Hosp/Harvard Med. Sch., Charlestown, MA; [2]Envrn. and Occup. Hlth. Sci. Institute, Univ. of Med. and Dent. of New Jersey, Piscataway, NJ |
| Abstract: | Parkinson's disease (PD), a progressive neurodegenerative disorder, results from the interaction of genetic and environmental factors in association with aging. From a genetic standpoint human α-synuclein (*hw-αSYN*) has been implicated in the PD phenotype. Mutations in the human α-synuclein gene (A53T, A30P, 3rd one) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human α-synuclein gene with two of these mutations have been shown to develop age-related reductions in number of dopaminergic nigral neuron, level of striatal dopamine (DA) and locomotor activity. Pharmacological blockade of the adenosine $A_{2A}$ receptor using caffeine (non-specific $A_{2A}$ antagonist) and specific antagonists, as well as genetic elimination of the $A_{2A}$ receptor in mice have all been shown to be neuroprotective in mouse models of PD. The present study sought to determine whether deletion of the $A_{2A}$ receptor ($A_{2A}$[-/-] mice) would afford protection against the age-related reduction of striatal |

Appendix I

dopamine in double-mutant human α-synuclein (*hm$^2$-αSYN*) mice. Heterozygous *$A_{2A}$*[+/-] mice were mated with *$A_{2A}$*[+/-] mice that were also transgenic for the wild-type (*hw-αSYN*) or doubly mutated (*hm$^2$-αSYN)* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter - all in the C57Bl/6J background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional $A_{2A}$ receptor, DA content was significantly lower (by 35%) in hm$^2$-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in *$A_{2A}$*[-/-] mice (lacking functional $A_{2A}$ receptor) the hm$^2$-αSYN transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender. Thus the $A_{2A}$ receptor appears to be required for neurotoxicity in this α-synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific $A_{2A}$ antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

Disclosures: **A. Kachroo** , None; **E.K. Richfield**, None; **M.A. Schwarzschild**, None.

Support: NIEHS Grant R01010804

DOD Grant W81XWH-04-1-0881

AFAR/Beeson program

[Authors]. [Abstract Title]. Program No. XXX.XX. 2008 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2008. Online.

Appendix J

Print this Page


Presentation Abstract

| | |
|---|---|
| Program#/Poster#: | 453.6/BB34 |
| Title: | Effect of conditional knockout of striatal adenosine A2A receptors on MPTP-induced nigrostriatal toxicity in mice |
| Location: | Washington Convention Center: Hall A-C |
| Presentation Time: | Monday, Nov 17, 2008, 2:00 PM - 3:00 PM |
| Authors: | ***A. PISANU**, M. SENA-ESTEVES, M. A. SCHWARZSCHILD; Neurol, Massachusetts Gen. Hosp., Charlestown, MA |
| Abstract: | Over the past decade large prospective epidemiologic studies have linked consumption of coffee and other caffeinated beverages to a reduced risk of developing Parkinson's disease (PD) later in life, raising the possibility that caffeine might protect dopaminergic neurons from degeneration in PD. Experimental evidence has shown that caffeine, as well as more specific antagonists of adenosine $A_{2A}$ receptors ($A_{2A}$Rs), attenuates neurotoxicity in experimental models of PD, and that MPTP-induced losses of striatal dopamine and dopamine transporters are significantly attenuated in $A_{2A}$R knockout (KO) mice compared to their wild-type littermate. But how and where $A_{2A}$R blockade or disruption influences the death of nigrostriatal dopaminergic neurons remains still uncertain. There is little evidence for appreciable expression of $A_{2A}$Rs on dopaminergic nigrostriatal neurons; whereas high levels are expressed on GABAergic striatopallidal output neurons, which are postsynaptic to the dopaminergic neurons that degenerate in PD. To better address the role of striatal $A_{2A}$R in neurotoxicity we took advantage of a viral Cre/*loxP* conditional KO system: infusion of an adeno-associated virus (AAV) vector encoding Cre |

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recombinase into the striatum of homozygous floxed $A_{2A}R$ ($A_{2A}R^{flox/flox}$) adult mice induces a selective depletion of $A_{2A}R$ in infected cells. Using this AAV-*cre*/*loxP* system, a double injection of AAV2/1-*cre* ($3x10^{13}$ gc/ml) into the rostral and caudal striatum on one side induced a 50% loss of striatal $A_{2A}R$ expression by day 30 post-injection. This focal $A_{2A}R$ KO phenotype remained stable at day 60 post-injection, as revealed by immunohistochemical detection of *cre* expression and absence of $A_{2A}R$ expression in the same area. Fifty days after infection, AAV2/1-*cre* injected $A_{2A}R^{flox/flox}$ mice and their respective AAV2/1-*GFP* controls were treated with MPTP (15mg/kg) or saline i.p every 2 hours for a total of 4 doses. Striatal Tyrosine hydroxylase (TH) immunoreactivity (IR), representing dopaminergic projections, was measured one week later. In control AAV2/1-*GFP*-injected mice, the loss of striatal dopamine was indistinguishable between ipsilateral and contralateral striata. By contrast, in AAV2/1-*cre*-injected mice the intensity of TH IR was higher in injected than in contralateral striatum. Further stereological analysis of surviving neurons in substantia nigra *pars compacta* is in progress - in order to confirm whether focal disruption of $A_{2A}R$ in the striatum could confer neuroprotection against MPTP toxicity in this model of PD.

Disclosures: **A. Pisanu** , None; **M. Sena-Esteves**, None; **M.A. Schwarzschild**, None.

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Appendix K

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Presentation Abstract

| | |
|---|---|
| Program#/Poster#: | 142.5/F14 |
| Title: | Adenosine $A_{2A}$ receptor gene deletion confers protection of nigral neurons in an α-synuclein model of Parkinson's disease |
| Location: | South Hall A |
| Presentation Time: | Sunday, Oct 18, 2009, 8:00 AM - 9:00 AM |
| Authors: | ***A. KACHROO**[1], E. K. RICHFIELD[2], M. A. SCHWARZSCHILD[1]; [1]Mass Gen. Hosp/Harvard Med. Sch., Charlestown, MA; [2]Envrn. and Occup. Hlth. Sci. Institute, Univ. of Med. and Dent. of New Jersey, Piscataway, NJ |

Abstract: Parkinson's disease (PD), a progressive neurodegenerative disorder, results from the interaction of genetic and environmental factors in association with aging. From a genetic standpoint human α-synuclein (*h-αSYN*) has been implicated in the PD phenotype. Mutations in the (h-αSYN) gene (e.g., A53T and A30P) may cause autosomal dominant familial PD. Transgenic mice expressing a *h-αSYN* gene with these two mutations (*hm*$^2$*-αSYN* mice) develop age-related reductions in dopaminergic nigral neurons, striatal dopamine (DA) and locomotor activity. Pharmacological blockade of the adenosine $A_{2A}$ receptor using caffeine (non-specific $A_{2A}$ antagonist) and specific antagonists, as well as genetic elimination of the $A_{2A}$ receptor are all neuroprotective in mouse models of PD. The present study sought to determine whether deletion of the $A_{2A}$ receptor ($A_{2A}$[-/-] mice) would afford neuroprotection in *hm*$^2$*-αSYN* mice. Heterozygous $A_{2A}$[+/-] mice were mated with $A_{2A}$[+/-] mice that were also transgenic for

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the wild-type (*hw-αSYN*) or doubly mutated (*hm$^2$-αSYN)* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase (TH) promoter - all in the C57B/6J background. Offspring were sacrificed at 20-24 months of age. Striatal DA and metabolites were measured by HPLC-ECD. Stereological counts were performed in the substantia nigra pars compacta. Alternate brain sections collected were also stained using double immunohistochemistry for both TH-positive neurons and synuclein to assess the levels of *h-αSYN* in nigral dopaminergic neurons from brain sections of $A_{2A}$[+/+] and $A_{2A}$[-/-] hm$^2$-αSYN male mice. In the presence of a functional $A_{2A}$ receptor, stereological counts were significantly lower (by approx 43%) in hm$^2$-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in $A_{2A}$[-/-] mice the hm$^2$-αSYN transgene did not reduce neuronal cell counts compared to either control. Striatal DA data showed a profile similar to that observed with the stereological counts. No differences were observed in the expression levels of *hm$^2$-αSYN* immunoreactivity between the two $A_{2A}$ genotypes, indicating that a reduction in *hm$^2$-αSYN* expression does not account for the reduced toxicity observed in the $A_{2A}$[-/-] hm$^2$-αSYN mice. Thus the $A_{2A}$ receptor appears to be required for neurotoxicity in this α-synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific $A_{2A}$ antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

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dendrites of medium-sized spiny neurons (MSNs) which contain high levels of NO receptors called soluble guanylyl cyclases (sGC). Recent studies have revealed that the effects of tonic and phasic NO-sGC-cGMP signaling on MSN activity are likely to be complex. It is becoming clear, however, that under physiological conditions transient elevations in intracellular cGMP primarily act to increase neuronal excitability and facilitate the short-term potentiation of excitatory corticostriatal transmission. NO-cGMP signaling also functionally opposes the inhibitory effects of DA D2 receptor activation on corticostriatal transmission. Not surprisingly, alterations in striatal NO-sGC-cGMP signaling are apparent following loss of DA innervation and may contribute to pathophysiological changes observed in basal ganglia circuits in Parkinson's disease (PD). In support of this, our current studies performed in DA-depleted rats show that acute pharmacological disruption of sGC-cGMP signaling reverses pathological elevations in: 1) single-unit activity recorded in the striatum, and 2) cytochrome oxidase staining measured in the subthalamic nucleus. Moreover, systemic administration of a selective sGC inhibitor transiently attenuated forelimb akinesia observed in both rat and mouse models of PD. These observations suggest that cGMP signaling contributes in an important manner to the overactive excitatory transmission observed in striatopallidal neurons in the DA-depleted striatum. Thus, down-regulation of the NO-sGC-cGMP pathway may represent an effective therapeutic strategy for restoring motor deficits observed in PD.

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## O-42
## THE ROLE OF SEROTONIN 2A RECEPTORS IN THE BASAL GANGLIA

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The dorsal raphe nucleus provides the major serotonergic input to the basal ganglia circuitary with projections to the striatum, the globus pallidus, the subthalamic nucleus, the substantia nigra, the pedunculopontine nucleus and the cerebral cortex, including the motor cortices. Serotonin (5-HT) has been shown to modulate dopaminergic neurotransmission in the striatum as well as GABA and glutamate neurotransmission in the output regions of the basal ganglia via a number of 5-HT receptors, suggesting a role for 5-HT in movement control. Of these, 5-HT1A, 5-HT1B, 5-HT2A and 5-HT2C receptors have been the subject of intense research for their potential in treating Parkinson's disease and other motor disorders. We investigated the mechanism underlying the reported antiparkinsonian action of the mixed 5-HT2A/2C receptor antagonist ritanserin. We compared the effects of ritanserin with the selective 5-HT2A receptor antagonist M100907 and the selective 5-HT2C receptor antagonist SB 206553 on motor impairments in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP-treated mice exhibited decreased performance on the beam-walking apparatus. These motor deficits were reversed by acute treatment with levodopa. Both the mixed 5-HT2A/2C antagonist ritanserin and the selective 5-HT2A antagonist M100907 improved motor performance on the beam-walking apparatus. In contrast, SB 206553 was ineffective in improving the motor deficits in MPTP-treated mice. In vivo microdialysis studies revealed that striatal extracellular glutamate levels were elevated in the MPTP-treated mice. Furthermore M100907 reduced striatal glutamate to near control levels in the MPTP mouse model of parkinsonism. The data suggest that 5-HT2A antagonists improve parkinsonian motor impairments probably by altering glutamatergic neurotransmission in the striatum. Blockade of 5-HT2A receptors may represent a novel approach to ameliorate motor symptoms of Parkinson's disease.

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## O-43
## PURINES AND DISEASES OF THE BASAL GANGLIA

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Endogenous purines --from adenosine to urate --have emerged as therapeutic targets for several basal ganglia disorders. Adenosine and its actions via its A2A receptor subtype have been intensively explored as an 'atypical' basal ganglia neuromodulator system contributing to the motor dysfunction of Parkinson disease (PD). Indeed A2A receptor antagonists are now a leading class of non-dopaminergic agents aimed at the motor symptoms of PD. A critical mass of at least 5 distinct A2A antagonists have entered clinical trials for PD. Contributing to the scientific interest and industry investment in these antiparkinsonian agents are unique features of the A2A receptor: 1) its remarkably restricted regional and cellular expression patterns in the CNS; 2) its capacity to form functional heteromeric receptor complexes with other GPCRs (e.g., dopamine D2, cannabinoid CB1, and mGlu5 receptors); 3) its possible involvement in other CNS symptoms (e.g., restless legs, dyskinesia, excessive daytime sleepiness, depression); 4) its potential role in the underlying neurodegeneration of PD. This is based on a convergence of epidemiological data linking caffeine (an adenosine antagonist) to a reduced risk of PD, with lab data demonstrating neuroprotection by A2A blockade.

Recently, another purine has emerged as a compelling candidate neuroprotectant in PD and possibly Huntington's disease (HD). Urate, a major antioxidant as well as the metabolic end product of adenosine in humans, displays neuroprotective properties in cellular models of PD. In humans higher levels of urate have been identified as the first molecular predictor of both a reduced risk of typical PD and a reduced rate of its progression. Similarly, higher urate levels have been linked to better outcomes among those with HD. We have begun a phase II clinical trial of a urate-elevating strategy using the purine intermediate inosine in an effort to rapidly translate these findings into disease-modifying therapy for PD.

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# Adenosine $A_{2A}$ Receptor Antagonists for Parkinson's Disease

## Rationale, Therapeutic Potential and Clinical Experience

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## Abstract

Long-term disability in Parkinson's disease (PD) is related to progression of the underlying disease and the emergence of complications of chronic levodopa therapy. There is a need for new medications that can slow the underlying progression of degeneration, improve PD symptoms in early disease without inducing dyskinesia, and improve motor fluctuations and 'off' time in advanced disease without worsening dyskinesia. Much interest has focused on the development of nondopaminergic therapies, with antagonists of the adenosine $A_{2A}$ receptor emerging as leading candidates. $A_{2A}$ receptors are selectively expressed in the basal ganglia and specific $A_{2A}$ antagonists reverse motor deficits without causing dyskinesia in animal models of PD. The antiparkinsonian potential of $A_{2A}$ receptor blockade has been expanded further by convergent epidemiological and laboratory findings suggesting a possible neuroprotective effect of $A_{2A}$ receptor antagonists in PD. Istradefylline (KW-6002) is the first of several adenosine $A_{2A}$ receptor antagonists in development for PD to advance to phase III clinical trials. Initial studies indicate that in patients with motor fluctuations on levodopa, addition of istradefylline reduces 'off' time. Additional studies are necessary to evaluate the benefit of istradefylline as monotherapy in early disease, its effect on the development of dyskinesia, and its effect on disease progression.



Although current medication treatment of Parkinson's disease (PD) provides good benefit for a number of years, long-term treatment remains inadequate. The underlying neuronal degeneration continues to progress and many patients develop long-term complications of dopamine replacement therapy.[1] Continued neuronal degeneration can lead to the emergence of dementia or imbalance, problems that can cause substantial disability and that are poorly responsive to symptomatic treatment. In addition, many patients develop fluctuations in response to treatment with levodopa and experience dyskinesia (twisting, turning movements) during peaks of levodopa-derived dopamine in the brain. Dyskinesia itself can be disabling,[2] and also commonly prevents the administration of additional dopaminergic medication to improve symptoms when levodopa has worn off ('off' time).

Because of these limitations of current therapy, an intense search for new medications to treat PD is ongoing. There is a need for medications that can slow the underlying progression of degeneration,

improve PD symptoms in early disease without inducing dyskinesia, and improve motor fluctuations and 'off' time in advanced disease without worsening dyskinesia. Much interest has focused on the development of nondopaminergic therapies, especially adenosine $A_{2A}$ receptor antagonists. Istradefylline (KW-6002) is an adenosine $A_{2A}$ receptor antagonist that is now in phase III clinical trials for PD.

## 1. Preclinical

### 1.1 Neurobiology of $A_{2A}$ Receptors

The case for developing adenosine $A_{2A}$ receptor antagonists as antiparkinsonian therapy has been built on a solid foundation of preclinical evidence. Here we review the distinctive neurobiology of this receptor, and then highlight findings in animal models of PD that have justified clinical trials of $A_{2A}$ receptor antagonists. For more comprehensive assessments, the reader should see Kase et al.[3] and Xu et al.[4]

Adenosine is a ubiquitous purine that plays multiple fundamental roles within all cells, conveying genetic information (as an elemental component of DNA and RNA), storing metabolic energy (in adenosine triphosphate [ATP]) and transducing cytoplasmic signals (through cyclic adenosine monophosphate [cAMP]). In addition, adenosine functions outside of neurons and other cells to carry information between them.

The sources of extracellular adenosine acting on CNS neurons are poorly understood, but vary depending on neuronal locale (synaptic vs ambient) and conditions (normal vs pathological). Under basal conditions, ambient adenosine in the fluid bathing neurons may mirror intracellular levels because of passive bidirectional adenosine transporters, and in turn may exert a homeostatic influence on neuronal function.[5] By contrast, transient synaptic elevations in adenosine occur locally during vesicular neurotransmission with the co-release of ATP and classical neurotransmitters. ATP released into the synaptic cleft is rapidly converted to adenosine by the extracellular ectonucleotidase pathway.

Adenosine modulates neuronal function in mammals through four G protein-coupled adenosine receptor subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. The $A_1$ and $A_{2A}$ subtypes bind adenosine with high affinity, are coupled to adenylate cyclase (negatively and positively, respectively) and are expressed in high levels in the brain. However, $A_1$ and $A_{2A}$ receptors differ dramatically in their CNS distributions. Whereas $A_1$ receptors are widely expressed throughout the brain, the $A_{2A}$ receptor is highly enriched in the basal ganglia[6] (figure 1a). The relatively selective expression of the $A_{2A}$ receptor also contrasts with the broad distribution of neurotransmitter receptors (e.g. acetylcholine and glutamate receptors), antagonists of which are established nondopaminergic antiparkinsonian drugs. Although striatal receptors likely mediate the symptomatic benefits of anticholinergic agents (e.g. trihexyphenidyl) and putative antiglutamatergic agents (e.g. amantadine), their often prohibitive cognitive adverse effects presumably result from blockade of the high levels of extrastriatal (particularly cortical) receptors for these drugs (figure 1a, outer columns). The relatively restricted expression of the $A_{2A}$ receptor may thus account for the low propensity for CNS adverse effects noted thus far for $A_{2A}$ receptor antagonists in PD patients.[7-9]

The CNS pattern of $A_{2A}$ receptor expression is notable not only for its relative restriction to the striatum (figure 1a), but also because of its distinctive cellular localisation within the striatum (figure 1b). Adenosine $A_{2A}$ receptor messenger RNA (mRNA) is richly expressed in the subpopulation of medium-sized spiny GABAergic neurons forming the 'indirect' striatal output pathway, part of the basal ganglia circuitry that is disrupted in PD (as schematised in figure 2, and reviewed in more detail by Albin et al.,[10] Kase et al.,[11] and Wichmann and DeLong[12]). In the striatum, $A_{2A}$ receptor mRNA co-localises with dopamine $D_2$ receptor mRNA[13] as well as preproenkephalin mRNA,[14] in the striato-pallidal neurons of the indirect pathway.

The molecular and cellular signals generated by $A_{2A}$ receptor stimulation are just beginning to be understood. Biochemically, it is clear that the $A_{2A}$

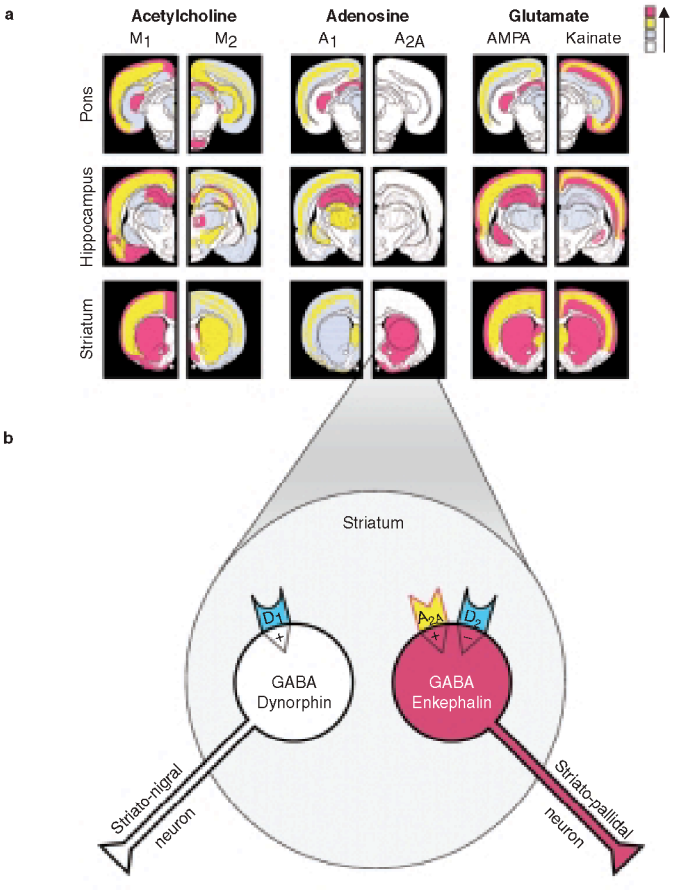


**Fig. 1.** Restricted regional and neuronal localisation of the adenosine $A_{2A}$ receptor in brain. (**a**) Brain expression of subtypes of three neurotransmitter receptors targeted for nondopaminergic therapy in Parkinson's disease. Composite distributions of specific radioligand binding to subtypes of muscarinic cholinergic ($M_1$ and $M_2$), adenosinergic ($A_1$ and $A_{2A}$) and ionotropic glutamatergic (1-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid [AMPA] and kainate) receptors are shown in coronal sections from the caudal, mid- and rostral rat brain (containing pons, hippocampus and striatum, respectively). Increasing density of radioligand-binding is colour-coded over a spectrum from white to blue to yellow to red. Most striatal receptors that modulate dopaminergic neurotransmission are also widely distributed throughout the brain, whereas the $A_{2A}$ subtype of adenosine receptor is relatively restricted in its expression to the striatum and the underlying olfactory tubercle (reproduced from Tohyama and Takatsuji[6] with permission from Oxford University Press and Igaku-Shoin Ltd). [**b**] Schematic of $A_{2A}$ receptor distribution at the cellular level between the two major types of output neurons of the striatum. Striatal $A_{2A}$ receptors are largely restricted to the GABAergic medium spiny neurons co-expressing enkephalin and dopamine $D_2$ receptors, and projecting to the globus pallidus. $A_{2A}$ receptors through G protein-coupled signalling pathways generally activate (+) neuronal activity, an influence that is counterbalanced by the inhibitory (−) effects of $D_2$ receptors on these neurons.

receptor can couple through stimulatory G proteins to activate adenylate cyclase, thereby enhancing production of the second messenger cAMP. By contrast, the $D_2$ receptor is negatively coupled to adenylate cyclase through an inhibitory G protein. These opposing actions of $A_{2A}$ and $D_2$ receptors co-localised to the striato-pallidal neuron (figure 1b) likely contribute to their counterbalancing behavioural as well as cellular effects.

The excitatory cellular effects of $A_{2A}$ receptor activation on striato-pallidal neurons are manifest as enhanced GABA release from their nerve terminals in the globus pallidus pars externa (figure 2).[15] Increased pallidal GABA then suppresses the inhibitory (GABAergic) pallidal projections to the subthalamic nucleus (STN), thereby activating (disinhibiting) the excitatory effects of glutamatergic STN projections to the globus pallidus pars interna

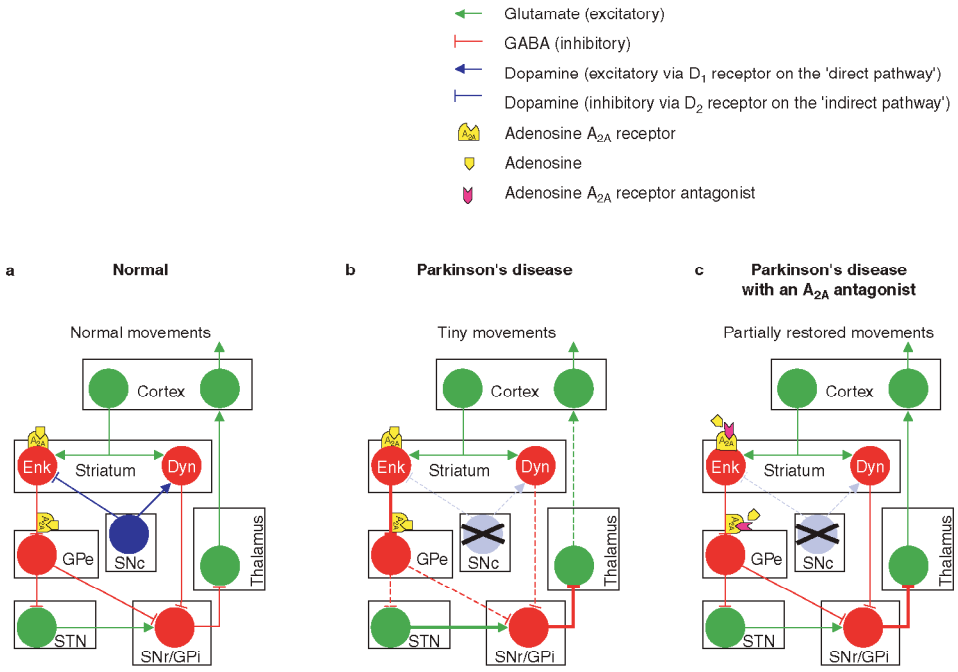


**Fig. 2.** Schematic of the probable mechanism by which $A_{2A}$ receptor antagonists relieve motor symptoms in Parkinson's disease (PD). As depicted in a simplified diagram of basal ganglia activity and its normal modulation of movement (**a**), dopamine acts at the striatal level to facilitate movement through stimulatory $D_1$ receptors on striato-nigral neurons of the 'direct' pathway and through inhibitory $D_2$ receptors on striato-pallidal neurons of the 'indirect' pathway. Adenosine acts on stimulatory $A_{2A}$ receptors on striato-pallidal neurons at postsynaptic sites in the striatum, and also at presynaptic sites on GABAergic nerve terminals in the globus pallidus pars externa (GPe). Loss of striatal dopamine in PD (**b**) disinhibits these striato-pallidal projection neurons, leading to GPe suppression and therefore disinhibition of the subthalamic nucleus (STN). Depletion of dopamine also leads to a parallel inhibition of striato-nigral neurons in the direct pathway. The resulting imbalance between the activities of the direct and indirect pathways in turn enhances inhibitory output from the internal GP (GPi) and substantia nigra pars reticulata (SNr) with excess inhibition of thalamo-cortical neurons, resulting in the characteristic reduced movement of PD. $A_{2A}$ receptor blockade in PD (**c**) should result in recovery of GPe activity. This in turn would reduce the excessive excitatory drive from the STN to the GPi/SNr complex, thereby partially restoring balance between the direct and indirect pathways. Accordingly, overactivity of GPi/SNr output neurons (and the resultant motor deficits) in PD may be partially reversed by $A_{2A}$ antagonists. Thickened or broken lines represent increased or decreased neural activity, respectively, along a pathway (reproduced from Albin et al.,[10] with permission; see also Xu et al.[4] and Kase et al.[11]). **Enk** = enkephalin; **Dyn** = dynorphin; **SNc** = substantia nigra pars compacta. **((Author: in our current house style we do not use variant font sizes. Is this suggested solution OK and, if not, do you have any other suggestions?))**

and substantia nigra pars reticulata. Activation of these GABAergic output nuclei of the basal ganglia leads to inhibition of the thalamic motor nuclei, which in turn leads to a reduction in the facilitative influence of thalamo-cortical activity onto the motor cortex. Through this mechanism, $A_{2A}$ receptors may contribute to motor inhibition in PD. Conversely, $A_{2A}$ antagonists partially reverse motor deficits through their inactivation of $A_{2A}$ receptors on striato-pallidal neurons. In support of this model, it has recently been demonstrated that the motor stimulant action of the $A_{2A}$ antagonist KW-6002 is eliminated after conditional knockout of the $A_{2A}$ receptor gene in neurons of the striatum.[16]

## 2. Influence of $A_{2A}$ Receptor Antagonists on Parkinson's Disease (PD) Pathophysiology

### 2.1 Symptomatic Actions

Although the anatomy of the $A_{2A}$ receptor distinguishes it from other nondopaminergic targets in the quest for improved antiparkinsonian therapy, it is the behavioural pharmacology of $A_{2A}$ receptor antagonists that has provided the central rationale for their development as antiparkinsonian agents. Relatively specific $A_{2A}$ receptor antagonists consistently reverse motor deficits or enhance dopaminergic treatments in animal models of PD. For example, in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the dopaminergic pathway, $A_{2A}$ receptor antagonists including SCH 58261, KW-6002 and MSX-3 all potentiated the contralateral turning behaviour induced by levodopa or a dopamine agonist.[17-20] In addition, motor stimulation by an $A_{2A}$ receptor antagonist in this model showed no tolerance after repeated treatment.[21]

Blockade of dopamine receptors with haloperidol and depletion of dopamine by reserpine are also used to model the hypodopaminergic motor dysfunction of PD. Under these conditions, rodents enter an akinetic cataleptic state, which can be reversed by the administration of $A_{2A}$ receptor antagonists.[22-24] Moreover, KW-6002 in combination with levodopa synergistically improves motor function in both haloperidol- and reserpine-treated rodents.[24]

Rigidity and rest tremor are also cardinal features of PD, and can be as disabling as bradykinesia. $A_{2A}$ blockade has been found to improve abnormalities of muscle tone and tremors in rodents, extending the potential benefit of $A_{2A}$ blockade for PD symptoms.[25-27] Again, combining an $A_{2A}$ receptor antagonist with levodopa produced a synergistic antiparkinsonian effect, in this case by alleviating the muscle rigidity induced in rodents by drugs that disrupt dopaminergic neurotransmission.[24]

With rodent studies suggesting the considerable potential of $A_{2A}$ receptor antagonists as symptomatic therapy for PD, evaluation of their efficacy was warranted in nonhuman primate models of PD. Initial investigations[28-30] confirmed the potential for facilitating motor activity while also demonstrating a low potential for inducing or exacerbating dyskinesias. In monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), KW-6002 produced a significant improvement in motor disability which persisted unabated over at least 3 weeks of treatment. Moreover, the observed reduction in motor disability scores was not accompanied by abnormal or excessive movements such as stereotypies or dyskinesia.[28,29] In the same animal model, KW-6002 was found to synergistically enhance the antiparkinsonian action of a low dose of levodopa or $D_2$ receptor agonist without exacerbating dyskinesia.[29,30] These latter studies helped establish the rationale for the initial clinical trials of an $A_{2A}$ receptor antagonist in patients with relatively advanced PD.

## 3. Disease-Modifying Potential

### 3.1 Neuroprotection

#### *3.1.1 Caffeine in the Epidemiology of PD*

Recent epidemiological findings have converged with laboratory studies to suggest that $A_{2A}$ receptor blockade by caffeine, a nonselective adenosine receptor antagonist, protects against the underlying neurodegeneration of PD. Coffee or tea drinking has

emerged as the dietary factor most consistently associated with a lowered risk of developing PD.[31] An inverse relationship between coffee drinking and PD was first demonstrated in case-control studies in the 1990s. Subsequent prospective studies strengthened the link between caffeine consumption and a reduced risk of later developing PD.[32,33] Thirty years after some 8000 Japanese-American men reported their dietary caffeine consumption, over 100 of them developed PD. Higher coffee intake at enrollment was dose-dependently associated with a reduced incidence of PD, including a >5-fold lower risk in those who drank over four medium (6oz) cups per day.[32] Confirmation of these findings was obtained in a prospective study of a larger, multi-ethnic population of men.[33] Coffee drinking, tea drinking, non-coffee caffeine consumption and total caffeine consumption were inversely correlated with the incidence of subsequent PD. By contrast, consumption of decaffeinated coffee was not associated with an altered risk of PD, clearly implicating caffeine as the component responsible for the inverse relationships.

Interestingly, comparison of the female and male cohorts by Ascherio et al.[33] exposed a stark gender difference, with no clear association between PD and caffeine intake apparent in women (as had been previously observed).[34] However, subsequent stratification of the women by estrogen exposure history in two independent prospectively followed cohorts[35,36] implicated a hormonal basis for this gender difference. In each study, in the subset of women who did not use postmenopausal estrogens, caffeine was significantly associated with a reduced risk of subsequent PD, just as in men.

#### *3.1.2 Neuroprotection by Caffeine and More Specific $A_{2A}$ Receptor Antagonists*

Despite their strengths, these epidemiological investigations cannot directly address the critical question: does caffeine prevent PD, or does early PD (or its causes) prevent the use of caffeine? Though the question of causality is difficult to answer in humans, animal models can offer useful clues. The hypothesis that caffeine protects humans from dopaminergic neurodegeneration has been strengthened by the finding that caffeine and more specific antagonists of the $A_{2A}$ receptor protect against dopaminergic neuron toxicity in rodent models of PD.

The effect of caffeine on the demise of nigrostriatal dopaminergic neurons has been investigated in the MPTP[37,38] and 6-OHDA[39] models of PD. Caffeine, when administered to mice at doses corresponding to those of typical human use, dose-dependently reversed the loss of striatal dopamine induced by MPTP.[37] Caffeine also prevented MPTP- or 6-OHDA-induced loss of dopaminergic nerve terminals in the striatum, and of dopaminergic cell bodies in the substantia nigra.[37,39,40]

The neuroprotective effects of caffeine appear to be mediated primarily by its antagonistic action at the $A_{2A}$ subtype of the adenosine receptor. MPTP- or 6-OHDA-induced nigro-striatal lesions were attenuated by all $A_{2A}$ receptor antagonists tested to date, including KW-6002[37,41,42] and SCH 58261.[37] Furthermore, $A_{2A}$ knockout mice (lacking functional $A_{2A}$ receptors as a result of targeted gene disruption)[43,44] were assessed for their susceptibility to dopaminergic neuron toxins. MPTP- (but not 6-OHDA-) induced losses of striatal dopamine and dopamine transporter binding sites were significantly attenuated in $A_{2A}$ knockout mice compared with their wild-type littermates.[37,45] Thus, complimentary genetic and pharmacological approaches have generally demonstrated that $A_{2A}$ receptor inactivation, like caffeine, can protect dopaminergic neurons. Overall, these data suggest that caffeine can protect against dopaminergic neuron injury and death through its antagonistic action at the adenosine $A_{2A}$ receptor. How the $A_{2A}$ receptor contributes to dysfunction and death of dopaminergic neurons remains unclear, but several plausible hypotheses[4] are being pursued.

### 3.2 Preventing Dyskinesias

A major impetus for developing novel nondopaminergic pharmacological approaches to PD has been the need to treat parkinsonian deficits without inducing or exacerbating dyskinesias. $A_{2A}$ receptor antagonists have indeed demonstrated a low potential for triggering dyskinesias in nonhu-

man primates. In addition, laboratory studies have raised the possibility that $A_{2A}$ receptor blockade may provide an additional benefit of preventing the development of dyskinesias in the first place.

Using a variety of rodent models of levodopa-induced dyskinesia in PD, several laboratories have evaluated the antidyskinetic potential of $A_{2A}$ receptor antagonists.[4] Some of these investigations suggest that ongoing $A_{2A}$ blockade may prevent the development of the maladaptive basal ganglia neuroplasticity that underlies dyskinesias.[21,45,46] Other studies that did not find evidence that $A_{2A}$ receptor antagonists can prevent the development of sensitised motor responses to L-dopa did, however, suggest that $A_{2A}$ blockade may reduce their expression – either directly[47] or indirectly (by allowing the use of lower levodopa doses to achieve the same antiparkinsonian benefit).[48]

To better assess the prophylactic antidyskinetic potential of $A_{2A}$ receptor antagonists, Bibbiani et al.[46] evaluated the primate model of dyskinesias. Monkeys previously lesioned (and made parkinsonian) by MPTP were treated daily with apomorphine (a short acting dopamine agonist) together with oral KW-6002 or vehicle for approximately 3 weeks, after which the daily treatments of apomorphine alone were continued for 3 more weeks. These investigators found that, in control animals, dyskinesias consistently developed 1–2 weeks after initiating dopaminergic treatment and persisted for the duration of the experiment. In contrast, monkeys that were initially treated for 3 weeks with dopamine agonist plus KW-6002 did not develop any abnormal movements during this co-treatment period, and only began to manifest dyskinesias 1–2 weeks after discontinuing the $A_{2A}$ receptor antagonist. This study supports a role for the $A_{2A}$ receptor in the development of dyskinesia (rather than in its expression) in primates, and provides additional rationale for targeting early PD patients in future human trials of $A_{2A}$ receptor antagonists.

### 3.3 Clinical Trials

Based on preclinical studies, adenosine $A_{2A}$ receptor antagonists have the potential to improve symptoms when taken as monotherapy in early PD and to reduce 'off' time without causing or worsening dyskinesia when taken as an adjunct to levodopa in advanced disease. Istradefylline (KW-6002) is now in phase III clinical trials for advanced PD, and results of several trials of this agent are available.

Bara-Jimenez et al.[7] at the US National Institutes of Health (NIH) conducted a 6-week, randomised, blinded, 'proof-of-principle' study using intravenous levodopa infusions to evaluate istradefylline in PD patients with moderate to advanced disease. Fifteen patients participated in the study. All were experiencing motor fluctuations on levodopa and six also exhibited peak-dose dyskinesias. Twelve patients were randomised to istradefylline treatment and three to placebo. One istradefylline subject withdrew from the study because of the need to take a prohibited medication. Subjects randomised to istradefylline received placebo for 2 weeks, istradefylline 40 mg/day for the next 2 weeks, and istradefylline 80 mg/day for the final 2 weeks. Efficacy evaluations were performed at the end of weeks 2 (baseline), 4, and 6. Oral antiparkinsonian medications were withheld on the night prior to the evaluation day and until assessments were completed. At each evaluation visit, function was assessed using the motor subsection of the Unified Parkinson's Disease Rating Scale (UPDRS) and the Abnormal Involuntary Movement Scale (AIMS). The effect of istradefylline administration was evaluated: (i) alone; (ii) with a suboptimal levodopa infusion; (iii) with an optimal levodopa infusion; and (iv) periodically following discontinuation of levodopa infusion. Results were analysed within active treatment subjects, comparing observations at weeks 4 and 6 to those at week 2.

When administered alone, istradefylline had no effect on parkinsonian signs and did not cause dyskinesias. Similarly, during optimal levodopa infusions, istradefylline had no effect on parkinsonian signs or dyskinesias. In contrast, during suboptimal levodopa infusions, istradefylline significantly improved parkinsonian signs. Motor UPDRS scores improved by 24% with istradefylline 40mg (from $30 \pm 2.8$ to $23 \pm 3.6$, $p < 0.05$) and by 36% with

istradefylline 80mg (from 30 ± 2.8 to 19 ± 4.3, $p < 0.02$). Of note, the antiparkinsonian response to istradefylline 80mg plus suboptimal levodopa infusion was similar to that produced by an optimal levodopa infusion, but with significantly less dyskinesia (45%, $p < 0.05$). Benefit was also identified for each of the individual cardinal signs, namely tremor ($p < 0.002$), rigidity ($p < 0.01$) and bradykinesia ($p < 0.05$). In addition, the time for the antiparkinsonian effect to decline by 50% following discontinuation of an optimal-dose levodopa infusion was increased by 76% ($p < 0.05$), from 62 ± 14 minutes to 109 ± 24 minutes. Istradefylline was relatively well tolerated. The most frequent adverse events were nausea (3), increased stiffness (3), headache (2) and increased dyskinesias (2).[7]

It is not clear why no antiparkinsonian benefit was identified when istradefylline was administered alone in this study and no other experience is currently available with istradefylline monotherapy in PD patients. It is possible that a higher istradefylline dose administered alone might provide antiparkinsonian benefit in this population. Perhaps more importantly, istradefylline monotherapy might provide benefit in early disease, when endogenous dopamine production and release are still present, although this has not yet been studied. The main findings of the NIH study, namely improved response when istradefylline is administered with a suboptimal levodopa infusion, and extension of the half-time of benefit following discontinuation of a levodopa infusion, suggest that istradefylline might be of benefit to reduce 'off' time in advanced patients with motor fluctuations, and possibly with reduced dyskinesia.

Hauser et al.[8] performed a 12-week, double-blind, placebo controlled, exploratory study of istradefylline as an adjunct to levodopa in PD patients with both motor fluctuations and dyskinesias (US-001 study). Patients were required to have at least 90 minutes of 'off' time between the first dose of levodopa after waking up for the day and 8 hours later to be eligible for the trial. Eighty-three subjects entered the study and were randomised to placebo, istradefylline 5 mg/day during weeks 1 to 4, 10 mg/day during weeks 5 to 8 and 20 mg/day during weeks 9 to 12 (5/10/20 group), or istradefylline 10 mg/day during weeks 1 to 4, 20 mg/day during weeks 5 to 8 and 40 mg/day during weeks 9 to 12 (10/20/40 group). Evaluations were undertaken at baseline and every 2 weeks through the study. Subjects completed 3 daily home diaries during the week before each visit.

Subjects randomised to istradefylline experienced a significant decrease in 'off' time compared with subjects randomised to placebo. Istradefylline recipients experienced a (mean ± standard error) reduction in the proportion of the awake day spent in the 'off' state of 7.1% ± 2.0% compared with an increase of 2.2% ± 2.7% in the placebo group ($p = 0.008$). Both istradefylline dose groups experienced a significant reduction in percent 'off' time compared with placebo. Similarly, subjects assigned to istradefylline experienced a reduction in 'off' time of 1.2 ± 0.3 hours compared with an increase of 0.5 ± 0.5 hours in the placebo group ($p = 0.004$). Thus, compared with placebo, istradefylline reduced 'off' time by 1.7 hours. This difference began to emerge at week 8 and was maximal at week 12, probably as a result of the escalating dose design of the study.

There were no significant differences in ratings of dyskinesia severity between the istradefylline and placebo groups. However, there was an increase in 'on' time with dyskinesia in the istradefylline group. It is difficult to determine the functional impact of this change because the diaries used in this study did not include such an assessment. Diaries that include the designation of troublesome and nontroublesome dyskinesia can help evaluate this effect and were therefore used in subsequent studies.[49]

Istradefylline was generally well tolerated. The most common adverse events in the istradefylline group were nausea (istradefylline 27.8% vs placebo 6.9%), aggravation of dyskinesia (16.7% vs 13.8%), dizziness (13.0% vs 3.4%) and vomiting (9.3% vs 6.9%). Nausea was mostly mild in intensity and usually resolved within 10 days.

This exploratory study suggested that istradefylline reduces 'off' time in patients with motor fluctu-

ations on levodopa and indicated that this should be the primary outcome variable in definitive studies of advanced PD patients. Severity of dyskinesia was not increased, although time 'on' with dyskinesia was increased.[8]

Two additional studies evaluating istradefylline as adjunctive therapy (US-005 and US-006) have been reported in abstract form.[9,50] These were 12-week, randomised, double-blind, placebo controlled studies of istradefylline 20, 40, or 60 mg/day in patients on levodopa with at least 2 hours of 'off' time on baseline diaries. In the 005 study, subjects were randomised to istradefylline 40 mg/day (n = 130) or placebo (n = 66); in the 006 study, subjects were randomised to istradefylline 20 mg/day (n = 163), istradefylline 60 mg/day (n = 155) or placebo (n = 77). In the 005 study, subjects assigned to istradefylline 40 mg/day experienced a (mean ± SD) reduction in 'off' time of 1.8 ± 2.8 hours compared with a reduction of 0.6 ± 2.7 hours in the placebo group ($p = 0.006$). Thus, compared with placebo, istradefylline provided a reduction of 'off' time of 1.2 hours. In the 006 study, there was a strong trend for greater reduction of percentage 'off' time in subjects assigned to istradefylline compared with placebo. The reductions in percent 'off' time were 7.9% ± 15.5% and 8.1% ± 14.2% in the istradefylline 20 mg/day and 60 mg/day groups, respectively, and 4.3% ± 17.2% in the placebo group ($p < 0.1$). When baseline percent 'off' time was included as a covariate, both istradefylline doses were found to provide a significant reduction in percent 'off' time compared with placebo ($p = 0.026$ and $p = 0.024$). 'On' time with nontroublesome dyskinesia increased with istradefylline in both studies but 'on' time with troublesome dyskinesia did not. The most common adverse events were increased dyskinesia (20 mg/day, 23.9%; 40 mg/day, 30.2%; and 60 mg/day, 21.5% vs 005 placebo 5.2%, and 006 placebo 14.3%) and nausea (20 mg/day, 10.4%; 40 mg/day, 10.9%; and 60 mg/day, 19.0% vs 005 placebo 9.1%, and 006 placebo 6.5%).[9,50]

Thus, clinical trials of istradefylline to date have provided rather consistent outcomes. In patients with motor fluctuations on levodopa, istradefylline reduces 'off' time by approximately 1.2 to 1.7 hours compared with placebo. In contrast to animal studies, there is some increase in dyskinesia but this increase appears to be an increase in nontroublesome dyskinesia and, therefore, may not have a negative impact. The most common adverse effect is nausea, occurring in 1–13% more patients receiving istradefylline than placebo. Nausea is generally mild and usually resolves within 10 days. Based on these clinical trials, istradefylline appears to be on track for approval as an adjunct to levodopa to reduce 'off' time in advanced PD patients.

## 4. Conclusion

In animal models, adenosine $A_{2A}$ receptor antagonists, either as monotherapy or as adjuncts to levodopa, have been demonstrated to improve parkinsonian signs without causing or worsening dyskinesia. This suggests that they may be useful as monotherapy in early PD to provide benefit without inducing dyskinesia and as adjuncts to levodopa in advanced disease to improve antiparkinsonian response without worsening dyskinesia. $A_{2A}$ receptor antagonists will be tested in early PD patients to assess antiparkinsonian efficacy and their ability to avoid or delay dyskinesia. In advanced PD, istradefylline (KW-6002) has been shown in preliminary studies to reduce 'off' time, with only a modest increase in dyskinesia. It is anticipated that other $A_{2A}$ receptor antagonists will soon enter clinical testing. In addition to symptomatic benefits, $A_{2A}$ receptor antagonists have been demonstrated to protect dopamine neurons *in vivo* from a variety of toxic insults. This suggests that they might have the potential to slow progression of PD and are candidates for clinical trials evaluating neuroprotection.

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Neurobiology of Disease

# Estrogen Prevents Neuroprotection by Caffeine in the Mouse 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Model of Parkinson's Disease

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**Epidemiological studies have strongly linked caffeine consumption with a reduced risk of developing Parkinson's disease (PD) in men. Interestingly, in women, this inverse association is present only in those who have not taken postmenopausal estrogens, suggesting an interaction between the influences of estrogen and caffeine use on the risk of PD. To explore a possible biological basis for this interaction, we systematically investigated how the neuroprotective effect of caffeine is influenced by gender, ovariectomy (OVX), and then exogenous estrogen in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD. (1) Caffeine treatment produced a dose-dependent attenuation of MPTP-induced striatal dopamine loss in both young and retired breeder (RB) male, but not female, mice. (2) In female mice (both young and RB), caffeine was less potent or altogether ineffective as a neuroprotectant after sham surgery compared to OVX or after OVX plus estrogen replacement compared to OVX plus placebo treatment. (3) Estrogen treatment also prevented the protection of caffeine against dopamine loss in young male mice. (4) Consistent with the putative protective effect of estrogen, female and OVX plus estrogen mice were relatively resistant to MPTP toxicity compared to male and OVX plus placebo mice, respectively. (5) There was no overall difference in brain levels of caffeine and its metabolites between OVX plus placebo and OVX plus estrogen mice. Together, these results suggest that estrogen can occlude and thereby prevent the neuroprotective effect of caffeine in a model of PD neurodegeneration, supporting a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.**

*Key words:* **adenosine $A_{2A}$ receptor; dopamine; gender; methylxanthine; ovariectomy; striatum**

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is pathologically well characterized. However, the etiology of PD remains unclear. Twin studies (Tanner et al., 1999; Vieregge et al., 1999; Wirdefeldt et al., 2004) have suggested that nongenetic factors, such as environmental exposures or random cellular events that occur during aging, play a prominent role in promoting the development of typical PD.

A major negative risk factor for PD has been identified recently as the consumption of caffeine. Multiple retrospective as well as several large prospective epidemiological studies have demonstrated that among dietary factors, previous coffee or tea drinking are consistently associated with a reduced risk of developing PD even after accounting for smoking and other potential confounding factors (Benedetti et al., 2000; Ross et al., 2000; Ascherio et al., 2001, 2004). The incidence of PD declines steadily with increasing intake of caffeine or coffee (but not decaffeinated coffee).

The mechanisms that underlie this epidemiological correlation remain unclear. One hypothesis that caffeine might represent a protective environmental factor in PD is supported by our findings that caffeine can protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal neurodegeneration in mice (Chen et al., 2001; Oztas et al., 2002). Moreover, the caffeine metabolites paraxanthine and theophylline provide similar attenuations of MPTP-induced dopaminergic toxicity (Xu et al., 2002b). In contrast to its locomotor stimulant effect, the neuroprotectant effect of caffeine does not show tolerance after chronic caffeine exposure (Xu et al., 2002a). Recently, the protection of caffeine against dopaminergic neuron loss and associated behavior changes was confirmed in the 6-OHDA rat model of PD (Joghataie et al., 2004). Together, the protective effects of caffeine and its metabolites in rodent models of PD support a causal basis for the inverse relationship between

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human caffeine consumption and the risk of subsequently developing PD.

Interestingly, the negative link between caffeine intake and risk of developing PD has been observed consistently in men but not in women (Benedetti et al., 2000; Ascherio et al., 2001, 2004). Overall, there was no clear relationship between PD and caffeine intake in two large prospectively followed populations of women (Ascherio et al., 2001, 2004). When these women were further divided by their estrogen use status after menopause, a negative association between caffeine intake and risk of PD similar to men was observed in those women who had never used estrogen replacement therapy but not in those who had ever used it (Ascherio et al., 2003, 2004). These results suggest that estrogen replacement therapy may prevent the beneficial effect of caffeine in reducing the risk of developing PD.

To address the possibility that estrogen, which can serve as a neuroprotectant in its own right, may interfere with neuroprotection by caffeine against dopaminergic neurotoxicity, we investigated their interaction in the MPTP mouse model of PD. We first assessed the difference in neuroprotection by caffeine in male and female mice. Then we systematically investigated the effect of exogenous estrogen on the neuroprotection of caffeine. Finally, we explored the effect of estrogen on the metabolism of caffeine as a possible mechanism of interaction between estrogen and caffeine.

## Materials and Methods

*Animals, ovariectomy, and estrogen replacement.* Young (~10 weeks old) or retired breeder (6–9 months old) male and female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used in these experiments. All experiments were performed in accordance with Massachusetts General Hospital and National Institutes of Health guidelines on the ethical use of animals. The mice were housed five per cage with *ad libitum* access to food and water and were maintained at a constant temperature and humidity with a 12 h light/dark cycle. To remove the main source of female hormones, bilateral ovariectomies were performed under anesthesia using Avertin (2% 2,2,2-tribromoethanol and 1% amyl alcohol; 20 ml/kg, i.p.) at either Charles River Laboratories or Massachusetts General Hospital. Sham operations were also included, in which all of the other procedures were the same, except for removal of the ovaries. In the experiments in which estrogen replacement was used, placebo or estrogen pellets (17$\beta$-estradiol, 0.1 mg per pellet, 21 d release; Innovative Research of America, Sarasota, FL) were implanted at the neck of mice under anesthesia 7–10 d after ovariectomy. This estrogen regimen was used because it produces an approximate replacement of physiological levels within the serum in ovariectomized (OVX) mice (Gao and Dluzen, 2001).

*Caffeine and MPTP treatment.* Different doses of caffeine (5, 10, 20, or 40 mg/kg) or saline were injected intraperitoneally 10 min before MPTP (40 mg/kg, i.p.) or saline injection ($n = 3$–7 for saline treatments and $n = 4$–15 for MPTP treatments). The extent of the caffeine dose range used across experiments varied depending on the number of mice available. In experiments in which ovariectomy and/or estrogen pellets were implanted, caffeine and MPTP treatments were performed 10 d after ovariectomy or estrogen implantation, whichever came later, to assure that estrogen levels were depleted or maintained at a constant level, respectively.

*Dopamine and 3,4-dihydroxyphenylacetic acid measurement.* One week after MPTP treatment, mice were killed by rapid cervical dislocation. The striatum was dissected out from the right cerebral hemisphere, frozen on dry ice, and stored at −80°C until use. Each striatum was weighed and extracted with 150 mM phosphoric acid and 0.2 mM EDTA. The striatum was homogenized and centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatants were analyzed for dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) content using standard reverse-phase HPLC with electrochemical detection (ESA, Chelmsford, MA). Biogenic amines were separated on a C-18 5 $\mu$m sphere column (Varian, Palo Alto, CA). The mobile phase consisted of 0.1 M sodium phosphate monobasic, 0.1 mM EDTA, 0.18 mM sodium octyl sulfate, and 8% methanol in filtered distilled water. The final pH of 3.3 was obtained with the addition of concentrated phosphoric acid, and the mobile phase was filtered and degassed before use. The dopamine and DOPAC contents were calculated as picomoles per milligram of tissue, and these values are presented within the figures as percentage of change from respective saline–saline-treated controls.

*Measurement of caffeine and its metabolites.* Ten days after estrogen or placebo pellet implantation, OVX retired breeder female mice were treated with saline or caffeine (5 or 40 mg/kg, i.p.; $n = 1$ for saline and $n = 5$ for caffeine). The mice were killed at 10, 30, 60, 120, 180, 240, or 360 min after injection by rapid cervical dislocation. The right cerebral hemisphere was dissected out, frozen on dry ice, and stored at −80°C until use. Each brain tissue was homogenized in 0.1 M monobasic sodium phosphate with a volume 10 times of tissue weight and centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatants were analyzed using liquid chromatography/mass spectrometry for determination of caffeine and its three metabolites, paraxanthine, theophylline, and theobromine. The lower limit of quantitation was 30 ng/ml. The analysis of caffeine and its metabolites was gratefully performed by Drs. R. L. Foltz and D. Andrenyak (Center for Human Toxicology, University of Utah, Salt Lake City, UT).

*Statistical analyses.* The data from striatal dopamine and DOPAC content as well as caffeine/metabolite measurements were analyzed by two-way ANOVA. *Post hoc* comparisons were performed using Fisher's least significant difference test. Data values in the figures represent the group mean ± SEM.

## Results

### Gender differences in the attenuation of MPTP toxicity by caffeine

The dose dependence of the neuroprotective effect of caffeine in the MPTP model of PD was first compared between intact male and female mice.

*Young mice*

MPTP treatment depleted the striatal dopamine level measured 1 week later down to 38% of control (saline treated) in ~10-week-old male mice (Fig. 1*A*). Caffeine pretreatment attenuated MPTP-induced dopamine loss in a dose-dependent manner in these young males, with a maximal effect (of doubling residual dopamine levels) achieved at 10 mg/kg. In female mice of the same age, MPTP depleted striatal dopamine levels to 62% of control levels (in saline-treated females). This reduction is significantly less than that observed in their male counterparts ($p < 0.05$), agreeing with previous reports (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998) that MPTP induced less dopaminergic toxicity in female than male mice. However, in contrast to male mice, female mice showed no attenuation of MPTP toxicity after lower doses (5, 10, or 20 mg/kg) of caffeine pretreatment. Only the highest dose (40 mg/kg) of caffeine protected against MPTP. Simultaneous measurement of DOPAC (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material), the major metabolite of dopamine in mouse, revealed that lower doses of caffeine pretreatment (10 or 20 mg/kg) significantly attenuated MPTP-induced depletion in male mice, whereas only the highest dose of caffeine pretreatment (40 mg/kg) provided similar protection in female mice.

*Retired breeders*

We also examined the neuroprotection of caffeine against MPTP toxicity in older (6–9 months old) retired breeder mice (Fig. 1*B*). Because these mice have passed their peak reproductive age, their comparison may be more relevant to gender differences in epidemiological studies of PD, which is usually diagnosed in people

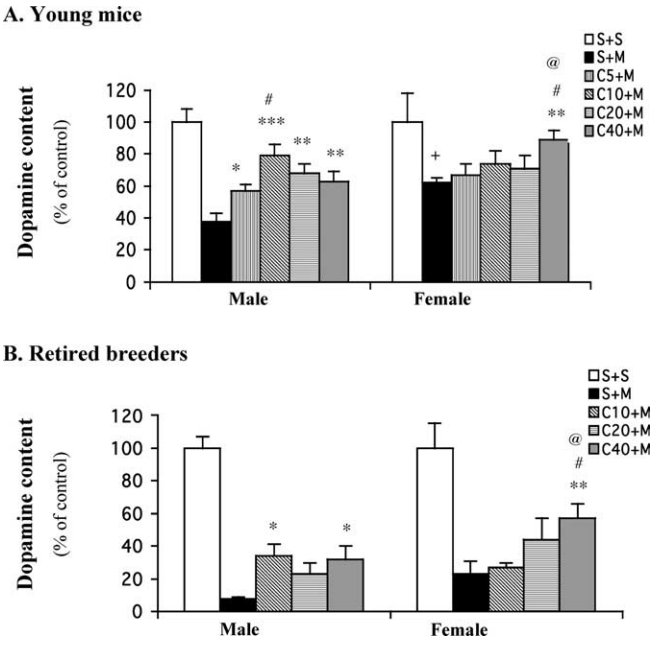


**Figure 1.** Caffeine dose-dependently attenuates MPTP-induced dopamine depletion in male but not female C57BL/6 mice, either young (~10 weeks old; ***A***) or retired breeder (6–9 months old; ***B***). Caffeine (5–40 mg/kg, i.p.) or saline was administered 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 4$–12) or saline ($n = 3$–6). One week later, striatal dopamine content was determined. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations (in picomoles per milligram of tissue) of these controls are 71.8 ± 2.4 and 70.6 ± 2.6 (***A***) and 80 ± 9 and 92 ± 9.8 (***B***) for males and females, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. ***A***, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.05$ compared with the respective C5+M group; $^{+}p < 0.05$ compared with S+M in male mice; $^{@}p < 0.01$ compared with C40+M in male mice. ***B***, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the respective S+M group; $^{\#}p < 0.05$ compared with the respective C10+M group; $^{@}p < 0.05$ compared with C40+M in male mice. S, Saline; M, MPTP; C5–40, caffeine at 5–40 mg/kg.

>50 years old. Not surprisingly, MPTP treatment induced a greater biochemical lesion in older mice for either gender (Fig. 1, compare *A*, *B*), as reported previously (Irwin et al., 1992). In male retired breeders, MPTP treatment depleted striatal dopamine levels to 8% of unlesioned controls (Fig. 1*B*). As in the younger male mice, caffeine pretreatment ≥10 mg/kg again significantly attenuated MPTP-induced dopamine depletion. In female retired breeders, MPTP treatment depleted striatal dopamine levels to 23% of control levels. Again, female retired breeder mice also showed less MPTP toxicity compared with that of male mice. However, just as in young mice, pretreatment with only the highest dose of caffeine (40 mg/kg) significantly attenuated the dopamine loss. Caffeine pretreatment, at all doses tested (10, 20, or 40 mg/kg), also attenuated loss of DOPAC in male mice, whereas only higher doses provided similar protection in female mice (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material).

### Ovariectomy increases the potency of the protective effect of caffeine

To investigate whether the gender difference in the neuroprotective effects of caffeine may relate to differences in female hormone status, we examined the effect of caffeine on MPTP toxicity in young female mice whose ovaries, the main site of female hormone production, were removed (OVX) or in sham-operated female littermates (Fig. 2). MPTP treatment depleted striatal

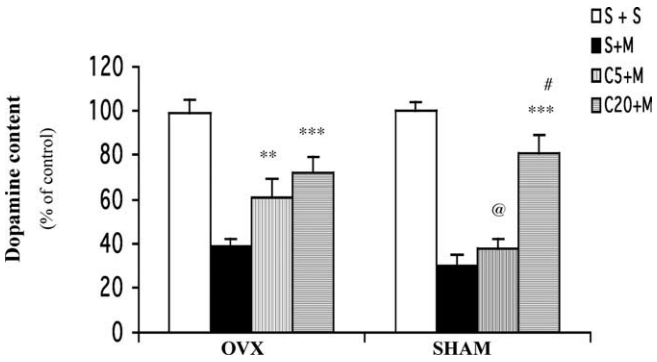


**Figure 2.** A low dose of caffeine attenuates MPTP-induced dopamine depletion in OVX but not sham-operated young female mice. Ten days after ovariectomy or sham operation, mice received caffeine (5 or 20 mg/kg, i.p.) or saline administration 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 8$) or saline ($n = 5$–7). One week later, striatal dopamine content was determined. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations of these controls are 61 ± 3.7 and 67 ± 2.7 pmol/mg of tissue for OVX and sham mice, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.001$ compared with the respective C5+M group; $^{@}p < 0.01$ compared with C5+M in OVX mice. S, Saline; M, MPTP; C5 or C20, caffeine at 5 or 20 mg/kg.

dopamine levels in both OVX and sham-operated mice. However, the lower dose of caffeine tested significantly increased residual striatal dopamine levels only in OVX females (Fig. 1, males). In contrast, sham-operated females (like intact females) required pretreatment with a higher dose of caffeine for attenuation of MPTP-induced dopamine loss.

### Estrogen attenuates the neuroprotection of caffeine in ovariectomized female mice

*Young mice*

To determine whether estrogen, the main female sex hormone, can account for the above-identified ovarian influence on neuroprotection by caffeine, we examined the effect of prolonged estrogen replacement on neuroprotection by caffeine in female mice depleted of endogenous estrogen (Fig. 3*A*). These OVX mice were implanted with subcutaneous pellets containing placebo or estrogen (17β-estradiol), which is continuously released to maintain steady-state concentrations for 21 d. MPTP treatment reduced striatal dopamine content to 27% of control in placebo-treated mice (OVX+P). The same MPTP exposure in OVX mice treated with estrogen pellets (OVX+E) depleted striatal dopamine levels, but to a significantly lesser extent (down to 39% of control), consistent with previous reports (Dluzen et al., 1996; Miller et al., 1998). In these OVX+P mice, pretreatment with caffeine, both at 10 and 20 mg/kg, significantly attenuated MPTP-induced striatal dopamine loss. In mice that were receiving estrogen replacement (OVX+E), however, only the higher dose of caffeine (20 mg/kg) provided significant protection. The striatal levels of DOPAC (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material) demonstrated a similar phenomenon.

*Retired breeders*

To better model estrogen replacement in the menopausal state, a parallel experiment was conducted in retired breeder (rather than young) mice (Fig. 3*B*). Again, estrogen by itself significantly attenuated MPTP-induced striatal dopamine loss. MPTP depleted dopamine levels to 23% of control in OVX+P mice, whereas it reduced them to 36% of control in OVX+E mice. In keeping with

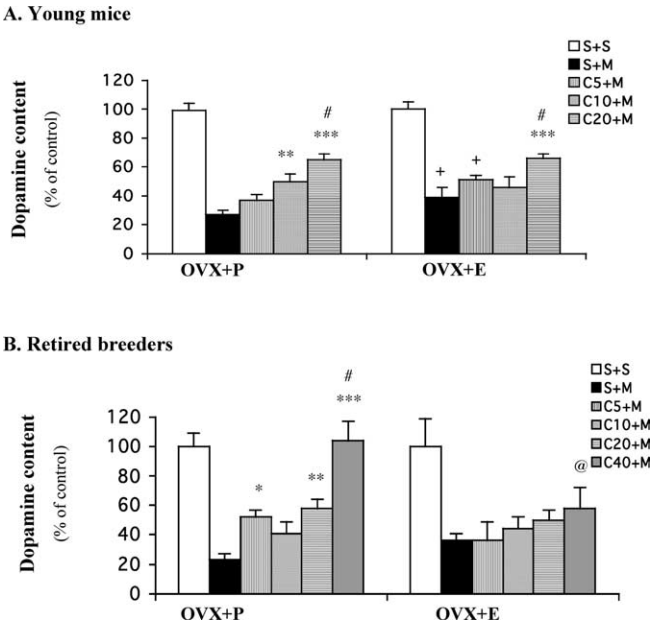


**Figure 3.** Caffeine attenuates MPTP-induced dopamine depletion in OVX female mice receiving placebo (OVX+P) but not estrogen (OVX+E) replacement (***A***, young mice; ***B***, retired breeders). Ten days after ovariectomy, mice were implanted with placebo or estrogen pellets (17$\beta$-estradiol, 0.1 mg per pellet, 21 d release) subcutaneously. Ten days later, caffeine (5–40 mg/kg, i.p.) or saline was administered 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 6$–15) or saline ($n = 3$–6). Striatal dopamine content was determined 1 week after MPTP. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations (in picomoles per milligram of tissue) of these controls are 72 ± 4 and 78 ± 4.7 (***A***) and 78.9 ± 7.3 and 73.9 ± 7.7 (***B***) for males and females, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. ***A***, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.05$ compared with the respective C5+M and C10+M groups; $^{+}p < 0.05$ compared with the respective S+M or C5+M in OVX+P mice. ***B***, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.001$ compared with the respective C5+M, C10+M, or C20+M group; $^{@}p < 0.05$ compared with C40+M in OVX+P mice. S, Saline; M, MPTP; C5–40, caffeine at 5–40 mg/kg.

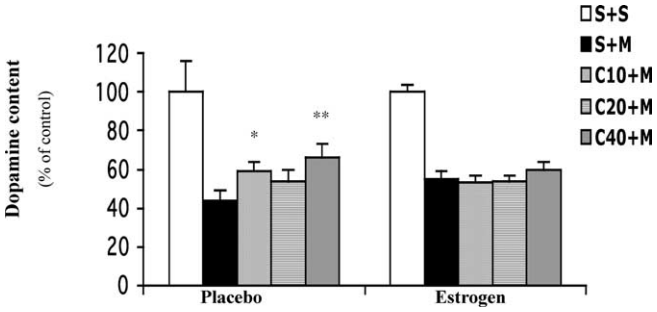


**Figure 4.** Caffeine attenuates MPTP-induced dopamine depletion in young male mice receiving placebo but not estrogen treatment. Mice were implanted with placebo or estrogen pellets. Ten days later, caffeine (10–40 mg/kg, i.p.) or saline was administered 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 8$–9) or saline ($n = 3$). Striatal dopamine content was determined 1 week after MPTP. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations of these controls are 79 ± 10.6 and 80.6 ± 4.2 pmol/mg of tissue for placebo- and estrogen-treated mice, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the respective S+M group. S, Saline; M, MPTP; C10–40, caffeine at 10–40 mg/kg.

observations in young mice, caffeine pretreatments (5, 20, or 40 mg/kg) significantly attenuated MPTP-induced dopamine loss in the OVX+P retired breeder mice, with complete reversal achieved at the highest dose of caffeine. However, in the setting of estrogen replacement in these OVX retired breeders, caffeine pretreatment did not confer protection at any dose tested. The measurement of DOPAC (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material) yielded a similar set of findings, with caffeine pretreatment (20 or 40 mg/kg) significantly reducing DOPAC loss in OVX+P but not OVX+E mice. Together, these results demonstrate that estrogen replacement can reduce or abolish the neuroprotective effect of caffeine on MPTP toxicity in young and older OVX females.

### Estrogen can prevent neuroprotection by caffeine in male mice

The above effects of manipulating endogenous and exogenous estrogen in female mice suggest that estrogen status may be the key factor accounting for the gender difference in the neuroprotective action of caffeine in the MPTP model of PD. To determine whether the neuroprotective effect of caffeine in males indeed relies on the absence or relatively low levels of estrogen, we assessed the effects of caffeine on MPTP toxicity in male mice implanted with placebo or estrogen pellets (Fig. 4). MPTP treatment depleted striatal dopamine levels to 44% and 55% of their respective control level in male mice pretreated for 21 d with placebo or estrogen, suggestive of a mild protective estrogen effect as repeatedly observed above. In the placebo-implanted males, caffeine pretreatments significantly attenuated striatal dopamine loss. However, no dose of caffeine pretreatment protected the male mice treated with estrogen pellets. These results demonstrate that estrogen can abolish the neuroprotection of caffeine not only in female mice but also in male mice.

### The effect of estrogen on caffeine metabolism in ovariectomized female mice

As a first step to understanding the mechanism of the influence of estrogen on neuroprotection by caffeine, we measured the brain levels of caffeine (trimethylxanthine) and its major demethylation metabolites (dimethylxanthines; i.e., paraxanthine, theophylline, and theobromine) at multiple time points after treatment with caffeine in OVX retired breeder female mice treated with placebo or estrogen pellets. Because estrogen status most consistently modulated the protective effect of lower doses of caffeine, we assessed the effect of estrogen on the CNS pharmacokinetics of caffeine administered intraperitoneally at 5 mg/kg (Fig. 5*A*). Brain concentrations of caffeine, which were maximal within 10 min of injection and declined with a half-life of ~50 min, were indistinguishable between the estrogen and placebo groups at all time points measured. When caffeine was administered at this low dose, the concentrations of its metabolites were below the limits of reliable detection. There was no measurable caffeine after saline injection.

As demonstrated above (Fig. 3*B*), in these OVX retired breeder mice, caffeine pretreatments attenuated dopamine depletion in placebo-treated but not in estrogen-treated mice, with the biggest difference found in the 40 mg/kg caffeine group. Therefore, we also determined brain concentrations of caffeine and its metabolites after intraperitoneal injection of caffeine at 40 mg/kg (Fig. 5*B*). There was no difference in brain caffeine concentrations between OVX+E and OVX+P mice. There was a small effect of estrogen on brain paraxanthine concentrations, which were slightly but significantly higher in OVX+P than in OVX+E mice at 120, 180, and 360 min. There was also a minor effect on brain theophylline concentrations, which were slightly higher in OVX+P than OVX+E mice 60 min after caffeine in-

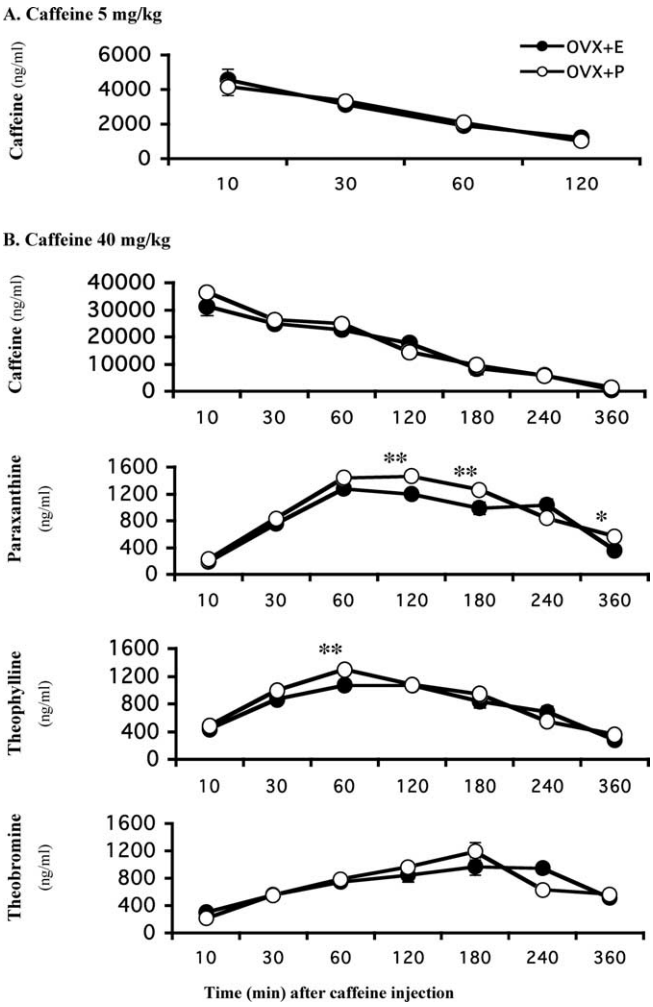


**Figure 5.** Caffeine metabolism is not changed by estrogen replacement in OVX retired breeder female mice. Ten days after ovariectomy, mice were implanted with placebo or estrogen pellets. Caffeine (5 or 40 mg/kg, i.p.) was administered 10 d after pellet implantation. Brain concentrations of caffeine and its metabolites were measured at 10, 30, 60, 120, 180, 240, or 360 min after injection ($n = 5$ for each time point). ***A***, Brain caffeine concentration after intraperitoneal 5 mg/kg caffeine. The levels of metabolites are below the detection limit. ***B***, Brain concentrations of caffeine, paraxanthine, theophylline, and theobromine, respectively, after intraperitoneal 40 mg/kg caffeine. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. *$p < 0.05$ and **$p < 0.01$ compared with the respective estrogen-treated group.

jection. There was no difference in brain theobromine concentrations after caffeine injection. Thus, exposure to estrogen, under conditions that attenuated the neuroprotective effect of caffeine, had no effect on brain concentrations of caffeine. Moreover, the overall concentrations of the three caffeine dimethyl metabolites even when combined are much (an order of magnitude) lower than that of caffeine such that the slight differences in metabolite concentrations found at some time points are not sufficient to explain the difference in caffeine neuroprotection between OVX+E and OVX+P mice.

## Discussion

The present data reveal a novel interaction between estrogen and caffeine in a mouse model of PD, an interaction that recapitulates the relationship between estrogen and caffeine exposures in the human epidemiology of PD. In male but not female mice, low doses of caffeine attenuated MPTP-induced depletion of striatal dopamine and DOPAC content, functional markers of the nigrostriatal neurons that degenerate in PD. Remarkably, neuroprotection by low doses of caffeine was abolished in the presence of estrogen (either endogenous or exogenous) in young and retired breeder OVX female mice. Moreover, estrogen placement in male mice effectively replicated the female phenotype of attenuated neuroprotection by caffeine.

### Estrogen treatment by itself protects against MPTP toxicity

Our data, in agreement with previous reports (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998), demonstrated that MPTP induced greater striatal dopamine loss in male than female mice. These experimental findings are consistent with epidemiological studies, which have generally demonstrated a higher prevalence of PD in men than women (Dluzen et al., 1998; Baldereschi et al., 2000; Ascherio et al., 2001, 2004). The gender differences observed in PD patients and animal models support the possibility that estrogen may play a protective role in dopaminergic neurodegeneration. Indeed, our current data confirm the neuroprotective effect of estrogen replacement therapy in this mouse model of PD (Dluzen et al., 1996; Callier et al., 2001; Ramirez et al., 2003).

### Attenuation of MPTP toxicity by caffeine is diminished in the presence of estrogen

The current data confirm our previous findings (Chen et al., 2001; Xu et al., 2002a,b) that caffeine dose-dependently attenuates MPTP-induced striatal dopamine loss in male mice. Moreover, our data demonstrate that this phenomenon persists as animals age. These findings support a biological basis for a causal epidemiological correlation between caffeine consumption and reduced risk of developing PD.

Strikingly, the protection by lower doses of caffeine against MPTP-induced dopamine depletion was lost in female mice. The gender difference in the dose–response relationship of caffeine with neuroprotection can be described pharmacologically as a reduction in the potency of caffeine in female compared with male mice. In both young and retired breeder mice (Fig. 1), an $ED_{50}$ value of $\leq$5 mg/kg is apparent in males compared with $\geq$20 mg/kg in females. As we discussed above, female mice are less vulnerable than males to MPTP toxicity, raising the possibility that attenuated protection by caffeine in females may also reflect a "ceiling effect" rather than reduced potency. However, the loss of protection in both moderate and severe lesions together with repeatedly significant protection achieved by the highest dose of caffeine in female mice of different ages argue against an impenetrable ceiling and support a lower potency in females. The current data correlate well with the epidemiological studies that showed no inverse association between caffeine or coffee intake and risk of PD in women (Benedetti et al., 2000; Ascherio et al., 2001). The neuroprotection observed in female mice was restricted to the highest caffeine dose of 40 mg/kg and may not be relevant to PD epidemiology. This dose in rodents may be comparable to a human exposure to the caffeine in some 10 cups of coffee (Fredholm et al., 1999), which exceeds the daily consumption of almost any human subject in PD epidemiology studies.

The most notable finding of the current study is that the presence of the major female steroid hormone estrogen prevents neuroprotection by caffeine (or reduces its potency) in female and male mice. The estrogen effects observed in young mice were more robust in mice past their reproductive prime, highlighting the relevance of these findings to epidemiological studies linking PD risk to caffeine and estrogen exposures of middle-aged adults. These data systematically demonstrate that estrogen treatment

attenuates the protection of caffeine against MPTP toxicity in this mouse model of PD, providing a possible biological basis for the epidemiological findings that an interaction between estrogen and caffeine exposure modulates the risk of PD (Ascherio et al., 2003, 2004).

**Mechanism of interaction between caffeine and estrogen**

In pursuing the mechanism, a pharmacokinetic or metabolic interaction is an important consideration. Human data (Patwardhan et al., 1980; Abernethy and Todd, 1985; Pollock et al., 1999) have demonstrated that caffeine metabolism is inhibited in women taking estrogen-containing oral contraceptives or estrogen replacement therapy after menopause, suggesting that the actions of caffeine would be enhanced rather than attenuated by estrogen. However, our data show no difference in brain caffeine concentrations over the time course (extending beyond three half-lives) of measurement in OVX retired breeder female mice treated with either estrogen or placebo.

Interestingly, two metabolites of caffeine (paraxanthine and theophylline) have been shown to provide protection against MPTP-induced dopaminergic toxicity with potencies comparable to caffeine (Xu et al., 2002b). So paradoxically, delaying or inhibiting the metabolism of caffeine could result in a decreased level of these metabolites, which might cause reduced protection against neurotoxicity after estrogen therapy in human or animals. In the present study, however, although there were slight differences in the brain concentrations of paraxanthine and theophylline between estrogen- and placebo-treated mice, the levels of these metabolites were much lower (>25 times) than that of caffeine, therefore arguing against their involvement.

It is interesting that although both estrogen and caffeine individually protect nigrostriatal dopaminergic neurons, when combined, estrogen diminished the potency (rather than enhancing the efficacy) of the neuroprotective action of caffeine. This finding suggests that these two agents may work through a common mechanism to prevent MPTP toxicity. Estrogen may compete with caffeine for its activation of a protective pathway, effectively "occluding" the pathway and shifting the dose–response curve of caffeine for neuroprotection to the right. It is worth noting that estrogen and caffeine as well as MPTP share a cytochrome P450, CYP1A2, for their metabolism or detoxification (Gu et al., 1992; Tassaneeyakul et al., 1994; Yamazaki et al., 1998; Forsyth et al., 2000). Therefore, metabolism of caffeine and/or estrogen could alter MPTP metabolism. However, because CYP1A2 contributes to hepatic detoxification of MPTP (Forsyth et al., 2000), competition for this enzyme by caffeine or estrogen would be expected to exacerbate rather than ameliorate MPTP toxicity. Moreover, because the brain MPTP levels were unaffected by acute caffeine treatment (Chen et al., 2001), it is unlikely that estrogen modulates the protection of caffeine through its action on MPTP metabolism. Alternatively, estrogen may noncompetitively inhibit the neuroprotective effect of caffeine while it provides its own independent protection effect. Our findings that higher doses of caffeine generally produce additional protection, however, argue against a simple noncompetitive inhibition.

Caffeine at the doses used in the present study, which produced peak brain concentrations of ~4–40 μg/ml, most likely functions as an antagonist of adenosine $A_1$ and $A_{2A}$ receptors (Fredholm et al., 1999). Our previous study (Chen et al., 2001) indicates that the attenuated neurotoxicity of caffeine likely involves its antagonism at the $A_{2A}$ receptor ($A_{2A}R$). Therefore, estrogen might directly modulate the neuroprotection by caffeine through an action on the $A_{2A}R$, although there is no information currently on such an interaction. It is reported (Rose'Meyer et al., 2003) that $A_{2A}R$ expression is significantly decreased after OVX. However, we found no difference in striatal $A_{2A}R$ binding densities between OVX mice treated with estrogen and placebo (data not shown). Among the potential mechanisms for neuroprotection by caffeine (and more specific $A_{2A}$ antagonists (for review, see Xu et al., 2005), the attenuation of glutamate release and of resultant excitotoxicity may be a protective mechanism shared with estrogen (O'Neill et al., 2004; Ritz et al., 2004; Saleh et al., 2004). Interestingly, it has been reported that estrogen can alter dopamine $D_2$ receptor ($D_2R$)/G-protein coupling (Levesque and Di Paolo, 1993; Thompson and Certain, 2005) and $D_2R$ affinity (Di Paolo et al., 1988). Because it is well known that $A_{2A}Rs$ colocalize with $D_2Rs$ in the basal ganglia and interact with each other closely (for review, see Xu et al., 2005), it is possible that estrogen could modulate $A_{2A}R$ through its action on $D_2R$. Whether these or other shared mechanisms of neuroprotection by caffeine and estrogen can account for their interaction in the MPTP model and in the epidemiology of PD remains to be clarified.

**Relevance of an estrogen–caffeine interaction for PD**

The attenuation of the neuroprotective effect of caffeine by estrogen in the MPTP model of PD establishes a potential neural basis for the epidemiological association between estrogen replacement therapy, caffeine consumption, and risk of developing PD in women. This convergence of laboratory and human epidemiological findings strengthens the contention that widely used exogenous estrogen, which may provide neuroprotection by itself, interacts with the nearly ubiquitously consumed $A_{2A}$ antagonist caffeine to modify the risk of developing PD. The present study also establishes an animal model of this interaction in humans, along with the opportunity to pursue its molecular mechanism(s). In addition, the cumulative evidence for this interaction is sufficiently compelling to influence the design and interpretation of neuroprotection trials of estrogen or caffeine currently underway or under consideration (Ravina et al., 2003). Ultimately, a better understanding of the interplay between environmental factors like caffeine and estrogen may suggest effective preventative as well as therapeutic strategies for this neurodegenerative disorder.

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Appendix B

Appendix O

Review TRENDS in Neurosciences Vol.29 No.11



# Targeting adenosine $A_{2A}$ receptors in Parkinson's disease

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**The adenosine $A_{2A}$ receptor has emerged as an attractive non-dopaminergic target in the pursuit of improved therapy for Parkinson's disease (PD), based in part on its unique CNS distribution. It is highly enriched in striatopallidal neurons and can form functional heteromeric complexes with other G-protein-coupled receptors, including dopamine $D_2$, metabotropic glutamate $mGlu_5$ and adenosine $A_1$ receptors. Blockade of the adenosine $A_{2A}$ receptor in striatopallidal neurons reduces postsynaptic effects of dopamine depletion, and in turn lessens the motor deficits of PD. $A_{2A}$ antagonists might partially improve not only the symptoms of PD but also its course, by slowing the underlying neurodegeneration and reducing the maladaptive neuroplasticity that complicates standard 'dopamine replacement' treatments. Thus, we review here a prime example of translational neuroscience, through which antagonism of $A_{2A}$ receptors has now entered the arena of clinical trials with realistic prospects for advancing PD therapeutics.**

## Introduction

Current pharmacotherapy for Parkinson's disease (PD) can be accurately described as both highly effective and largely inadequate. On the one hand, the dramatic efficacy of standard anti-parkinsonian drugs can be the stuff of Hollywood movies, as when the body of an immobilized patient played by Robert De Niro is 'awakened' by L-dopa in the 1990 film *Awakenings*. The characteristic bradykinesia (slowness), rigidity and tremor of PD are primarily due to an underlying degeneration of dopaminergic nigrostriatal neurons and the resultant depletion of striatal dopamine. Repleting endogenous dopamine using its precursor L-dopa and mimicking it using dopamine agonists constitute 'dopamine replacement' strategies – the mainstay of current treatment. By boosting dopamine-mediated transmission, these strategies can dramatically (albeit partially) alleviate the motor deficits in PD.

On the other hand, none of the currently approved anti-parkinsonian agents has been found to alter the underlying degeneration of dopaminergic neurons. Thus, after several years of gratifying improvement using dopamine-replacement therapy, PD patients typically experience great frustration as neuronal loss and symptoms inexorably progress. As the disease advances, disability is often compounded further by the development of L-dopa-triggered involuntary jerking and writhing movements known as dyskinesias. Eventually, most will also suffer from non-motor complications of both the dopaminergic treatments and the disease itself; such complications include sleep disturbance, depression, dementia and psychosis.

The inadequacies and adverse effects of drugs that target the dopaminergic system have prompted a search for alternative or adjunctive approaches that can modulate basal ganglia motor circuitry with a reduced risk of side effects. Antagonists of adenosine $A_{2A}$ receptors have recently emerged as a leading candidate class of non-dopaminergic anti-parkinsonian agents, based in part on the unique CNS distribution of the $A_{2A}$ receptor (Figure 1a). As we review here, promising preclinical features have not only led to clinical trials of $A_{2A}$ receptor antagonists as a novel symptomatic therapy for PD, but also raised the possibility of neuroprotective and anti-dyskinetic benefits.

## $A_{2A}$ antagonists as symptomatic anti-parkinsonian therapy

Neurochemical evidence that $A_{2A}$ receptors functionally oppose the actions of dopamine $D_2$ receptors on GABAergic striatopallidal neurons [1–3] (Figure 1b,c; Box 1) raised the possibility that $A_{2A}$ antagonists might boost the anti-parkinsonian action of dopamine-replacement strategies. Indeed, behavioral studies of hemi-parkinsonian rats, in which the dopaminergic nigrostriatal pathway had been lesioned on one side by 6-hydroxydopamine (6-OHDA), revealed that blockade of $A_{2A}$ receptors markedly increased the number of contralateral rotations induced by a threshold dose of L-dopa or by stimulation of dopamine receptors; Fos-like immunoreactivity in the dorsal striatum and globus pallidus was also increased [4–7]. These results provided the first indication that blockade of $A_{2A}$ receptors, by potentiating dopamine transmission, might contribute to restoring the motor impairment observed in models of PD. Importantly, in contrast to the non-specific adenosine antagonist caffeine, which can lose its motor-stimulant effect with repeated exposure, $A_{2A}$ antagonists did not produce tolerance effects after sub-chronic treatment in PD models [8,9].

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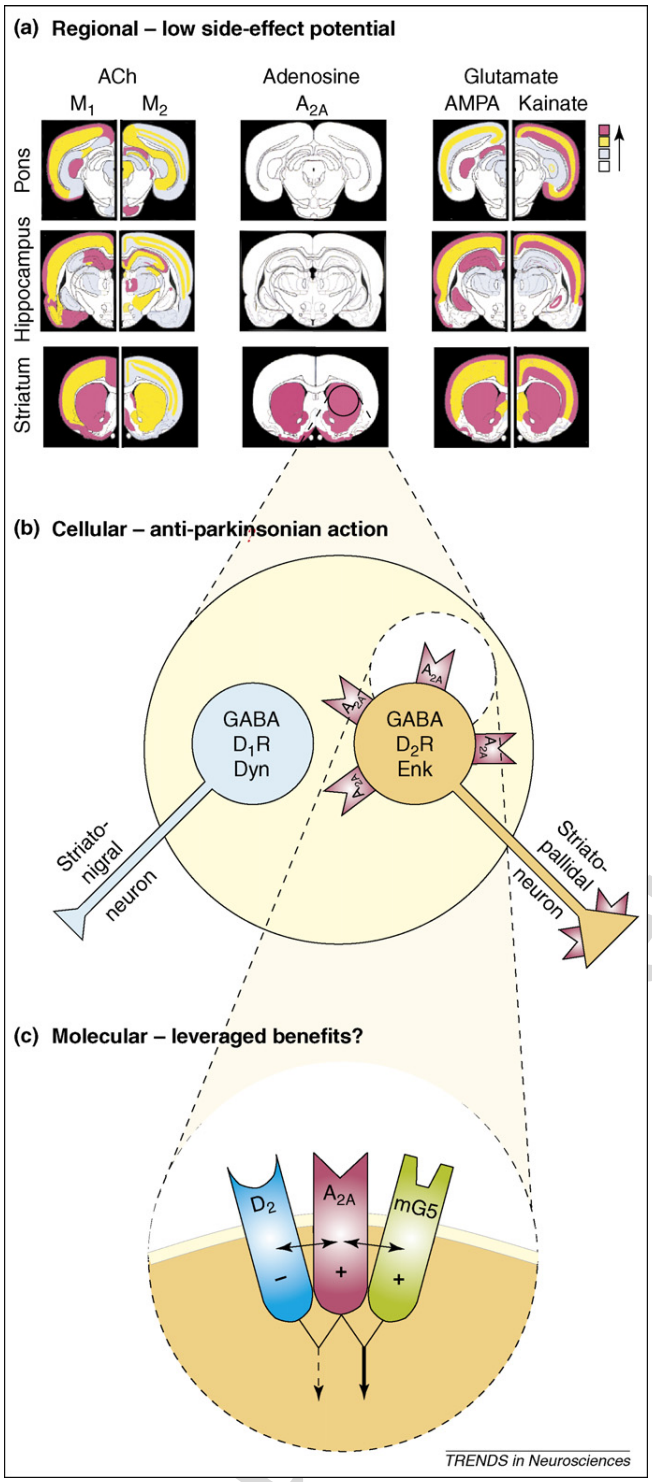


**Figure 1**. Unique CNS localization of adenosine $A_{2A}$ receptors at multiple levels contributes to the therapeutic potential of $A_{2A}$ antagonists for PD. **(a)** Regional localization of brain $A_{2A}$ adenosine receptors versus other non-dopamine receptors that are targeted in PD: the basis for the relatively low risk of CNS side effects from $A_{2A}$ antagonists. Brain expression patterns are shown for subtypes of receptors for three neurotransmitters that modulate dopamine-mediated neurotransmission. $A_{2A}$ receptors are highly enriched in the striatum, with very low (but possibly functionally significant) levels in the cortex and other brain regions. By contrast, other receptors targeted by current non-dopamine anti-parkinsonian drugs are widely distributed in the brain. Agents that block muscarinic ACh receptors and amantadine, which blocks the function of ionotropic glutamate receptors, improve PD symptoms but are often intolerable owing to cognitive and other CNS side effects, as would be expected from the broad expression of these receptors in the cortex and other extra-striatal brain regions. Increasing density of specific radioligand binding to each receptor subtype is represented by the color scale on the far right, and is shown in coronal sections from the caudal, mid and rostral rat brain (at the levels of the pons, hippocampus and striatum, respectively). Adapted, with permission, from Refs [13,73]. **(b)** Cellular localization of $A_{2A}$ receptors within the striatum: the basis for anti-parkinsonian action of $A_{2A}$ antagonists. In the striatum, $A_{2A}$ receptors are largely restricted to GABAergic neurons that express dopamine $D_2$ receptors ($D_2R$) and enkephalin (Enk) [17,18] and project to the globus pallidus. By contrast, the striatal GABAergic neurons that project to the substantia nigra are enriched for $D_1$ receptors ($D_1R$) and dynorphin (Dyn) but do not appreciably express $A_{2A}$ receptors. Inhibition of the striatopallidal neurons by $A_{2A}$ antagonism likely reduces motor deficits caused by dopamine deficiency in PD (Figure 2). **(c)** Molecular environment of $A_{2A}$ receptors: a possible opportunity for leveraging anti-parkinsonian effects. $A_{2A}$ receptors activate adenylate cyclase and generally stimulate neuronal activity. They can form heteromers with inhibitory ($G_i$-coupled) $D_2$ receptors [55,56,58] and stimulatory metabotropic glutamate $mGlu_5$ receptors (mG5) [65] to attenuate (thin broken downward arrow) or amplify (thick solid downward arrow) their respective signaling pathways. Functional heteromeric interactions can be direct (bidirectional arrows) or indirect (through downstream signaling, as indicated), and can open new avenues for enhancing anti-parkinsonian actions of $A_{2A}$ antagonists (Box 1). The interactions might occur postsynaptically, as schematized here on striatopallidal neurons, or presynaptically to modulate neurotransmitter release [66].

The ability of $A_{2A}$ antagonists to reverse parkinsonian deficits consistently in non-human primates [8,10] in addition to rodents led to clinical trials in PD patients that had encouraging, if modest, initial results [11,12] (reviewed in depth in Ref. [13]). These studies demonstrated symptomatic improvement in patients with relatively advanced PD, who had already developed dyskinetic motor complications. Pairing KW-6002, a well-characterized xanthine-based $A_{2A}$ antagonist, with a reduced dose of L-dopa produced symptomatic relief comparable to that produced by an optimal dose of L-dopa alone but with less dyskinesia [11]. However, when paired with a full dose of L-dopa, KW-6002 improved symptoms further only under some circumstances and perhaps slightly exacerbated dyskinesia [11–13].

Like bradykinesia, two other cardinal motor features of PD might also improve with $A_{2A}$ blockade. Muscle rigidity, manifest clinically as increased resistance to passive movement, is an early and progressive sign of PD. In rodents, muscle rigidity induced by the dopamine-depleting agent reserpine can be reduced by an $A_{2A}$ antagonist or eliminated by a synergistic combination of L-dopa plus $A_{2A}$ antagonist [14]. Parkinsonian rest tremor, which is relatively resistant to dopamine-replacement therapy, might also be targeted by $A_{2A}$ antagonists. In rodent models of parkinsonian tremor, $A_{2A}$ antagonists counteracted tremulous movements [15,16], in agreement with data from a recent clinical trial indicating that the combination of the $A_{2A}$ antagonist KW-6002 and a subthreshold dose of L-dopa counteracted resting tremor more effectively than they did other cardinal symptoms of PD [11]. Moreover, the intrastriatal infusion of the water-soluble non-xanthine $A_{2A}$ antagonist SCH BT2 demonstrated that, although the effects of $A_{2A}$ antagonists on tremor are exerted throughout the whole striatum, the ventrolateral portion is the area most involved in this effect [16].

The mechanism by which $A_{2A}$ antagonists improve parkinsonian motor dysfunction probably involves their direct inhibitory influence on striatopallidal neurons, which coexpress $A_{2A}$ and $D_2$ receptors [17,18]. The blockade of $A_{2A}$ receptors on these neurons offsets the hypolocomotor effect of lost dopamine stimulation at striatal $D_2$ receptors (Figure 2), accounting for much of the symptomatic

## Box 1. Receptor heteromerization: a key advance in $A_{2A}$ neurobiology

A fuller understanding of the biology of the $A_{2A}$ receptor, particularly its molecular interactions with other neurotransmitter receptors, will refine our ability to target it for the treatment of PD and other CNS disorders. The discovery of functional heteromeric receptor complexes (receptor mosaics) comprising the $A_{2A}$ receptor and other G-protein-coupled receptors (Figure 1c of the main text) constitutes a major advance in adenosine neurobiology and creates new opportunities to leverage the anti-parkinsonian actions (both symptomatic and disease-modifying) of $A_{2A}$ antagonists [52,53].

### $A_{2A}$–$D_2$ heteromers

Coimmunoprecipitation studies have demonstrated the existence of constitutive $A_{2A}$–$D_2$ complexes at the sites of colocalization [52,54,55]. Direct physical evidence for $A_{2A}$–$D_2$ heteromers in addition to $A_{2A}$ homodimers within the plasma membrane came from fluorescent and bioluminescent resonance energy transfer (FRET and BRET) analyses, indicating that $<10$ nm separates the receptors [56–58]. The stoichiometry of the $A_{2A}$–$D_2$ heteromers remains unknown and they could be dimers or higher-order $A_{2A}$–$D_2$ hetero-oligomers, which might help to explain antagonistic membrane-level (and ultimately behavioral) interactions between the $A_{2A}$ and $D_2$ receptors. For example, an $A_{2A}$–$D_2$ trimer containing a $D_2$ receptor dimer and an $A_{2A}$ monomer could account for the observed increase in the $K_d$ value of $D_2$ agonist binding sites if $A_{2A}$ monomer activation were to enhance negative cooperativity between the dimeric $D_2$ components [53,54]. $A_{2A}$ receptor activation can produce this direct molecular inhibition of $D_2$ receptors and might also reduce $D_2$ coupling to $G_i$ [52], suggesting that molecular interactions between $A_{2A}$ and $D_2$ receptors are important for the anti-parkinsonian actions of $A_{2A}$ antagonists. However, the fact that $A_{2A}$ antagonists improve parkinsonian motor deficits in the absence of the $D_2$ receptor (albeit to a lesser extent than in its presence) [59,60] indicates that a mechanism other than $A_{2A}$–$D_2$ heteromerization also contributes to their anti-parkinsonian properties.

### $A_{2A}$–$D_3$ heteromers

Similarly, evidence for functional $A_{2A}$–$D_3$ heteromers has recently been obtained in cells cotransfected with $A_{2A}$ and $D_3$, using FRET and by activating $A_{2A}$ receptors, which reduced both the affinity of $D_3$ agonist-binding sites and $D_3$ signaling [61]. Because $D_3$ dimers and tetramers exist in the brain [62], one can envisage the existence of higher-order $A_{2A}$–$D_3$ hetero-oligomers, in which cooperativity between multimeric $D_3$ components might be modulated by an $A_{2A}$ receptor within the receptor mosaic.

### $A_{2A}$–$mGlu_5$ heteromers

$A_{2A}$ receptors have also been found to partner with non-dopamine receptors – most notably the metabotropic glutamate receptor $mGlu_5$, which itself is candidate target for new symptomatic and neuroprotective anti-parkinsonian therapies. Evidence that $A_{2A}$ and group I mGlu receptor agonists, including $mGlu_5$ agonists, could synergistically reduce the affinity of the $D_2$ agonist-binding sites in striatal membranes [63,64] was followed by physical (coimmunoprecipitation) evidence that $A_{2A}$ and $mGlu_5$ form heteromeric receptor complexes [65]; such complexes might explain the synergy between $A_{2A}$ and $mGlu_5$ antagonists. This was supported by observations of a high degree of $A_{2A}$ and $mGlu_5$ colocalization in striatal neurons in primary cultures [52] (Figure Ia of this box), and more recently in glutamatergic nerve terminals in the striatum [66]. Coactivation of the $A_{2A}$ and $mGlu_5$ receptors caused a synergistic interaction at the level of *c-fos* expression and phosphorylation of extracellular signal regulated kinase (ERK) and dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), indicating a role for $A_{2A}$–$mGlu_5$ complexes in striatal plasticity [65,67]. Combined $A_{2A}$ and $mGlu_5$ receptor activation can also produce synergistic cellular effects on striatal output neurons *in vivo* (Figure 1c of the main text), as demonstrated by a greater than additive increase in GABA release from ventral striatopallidal neurons after local perfusion of both $A_{2A}$ and $mGlu_5$ agonists (Figure Ic of this box) [68].

### $A_{2A}$–$D_2$–$mGlu_5$ heteromers as therapeutic targets for PD

The discovery of heteromeric $A_{2A}$–$D_2$ complexes has added to the substantial evidence for antagonistic molecular, cellular, electrophysiological and behavioral interactions between $A_{2A}$ and $D_2$ receptors, and strengthens the rationale for anti-parkinsonian strategies that simultaneously block adenosine $A_{2A}$ receptors and stimulate dopamine ($D_1$ and $D_2$) receptors [52,54]. In retrospect, the initial clinical efficacy of $A_{2A}$ antagonism when tested as an adjunct to standard dopamine therapy (but not when tested as a monotherapy) in relatively advanced PD [11] might reflect the benefits of dual targeting. Notably, one might predict the greater likelihood of success using $A_{2A}$-antagonist monotherapy in earlier disease, when higher levels of residual endogenous dopamine provide greater tonic $D_2$ receptor activation.

Similarly, the discovery of functional $A_{2A}$–$mGlu_5$ interactions and heteromeric complexes catalyzed research into the synergistic anti-parkinsonian potential of combining $A_{2A}$ and $mGlu_5$ antagonists [69,70] (Figure Ic of this box). The additional finding that $mGlu_5$-antagonist-induced motor activation requires $A_{2A}$ and $D_2$ receptors [70] highlights the interdependence of these three receptors in modulating motor function. It also supports the concept of $A_{2A}$–$D_2$–$mGlu_5$ receptor mosaics [52,71]. These might integrate multiple signals converging on striatopallidal GABAergic neurons, and suggests future opportunities for leveraging the anti-parkinsonian actions of $A_{2A}$ antagonists.

Lastly, there is now physical and functional evidence that heteromeric $A_1$–$A_{2A}$ receptors can form both in receptor-overexpressing cell lines and in the striatum [72]. This study suggests that, just as in $A_{2A}$–$D_2$ receptor interactions, negative cooperativity could explain why $A_{2A}$ receptor stimulation might reduce $A_1$ receptor binding of adenosine, and thus reduce $A_1$-mediated inhibition of presynaptic glutamate release. Conversely, through such an interaction an $A_{2A}$ antagonist might increase binding of adenosine to its $A_1$ receptor, and thus amplify the inhibition of glutamate release, potentially alleviating excitotoxic injury to dopaminergic neurons.

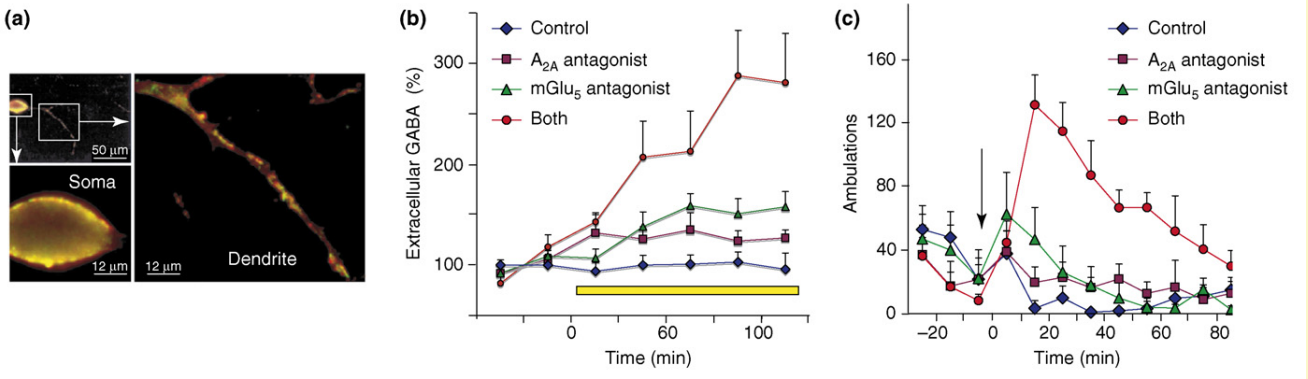


**Figure I.** $A_{2A}$–$mGlu_5$ receptor interactions in striatal neurons: anatomical, neurochemical and antiparkinsonian features. (a) $A_{2A}$ (green) and $D_2$ (red) receptor immunoreactivities are colocalized (yellow) and primarily associated with the plasma membrane in cell bodies and dendrites of striatal neurons in primary cultures.

Adapted, with permission, from Ref. [52]. **(b)** Combined activation of $A_{2A}$ and $mGlu_5$ receptors can synergistically activate striatopallidal neurons *in vivo*, as shown by the synergistic elevation of extracellular GABA levels (measured as a percentage of the average initial two baseline determinations) in the ventral pallidum of awake, freely moving rats after perfusion of both an $A_{2A}$ and an $mGlu_5$ agonist (CGS 21680 and CHPG, respectively) into the ventral striatum (indicated by the horizontal yellow bar). Adapted from Ref. [68]. **(c)** Combined antagonism of $A_{2A}$ and $mGlu_5$ receptors can synergistically enhance anti-parkinsonian locomotor stimulation in hypodopaminergic, hypolocomotor mice (treated one day earlier with 1 mg $kg^{-1}$ reserpine). Adapted from Ref. [70]. Mice were treated systemically (arrow) with control vehicles, an $A_{2A}$ antagonist (intraperitoneal KW-6002 at 0.3 mg $kg^{-1}$), an mGlu5 antagonist (intraperitoneal MPEP at 5 mg $kg^{-1}$) or both.

anti-parkinsonian benefit of $A_{2A}$ antagonism. However, this relatively selective targeting of the $D_2$-modulated 'indirect' pathway (Figure 2 legend) might also explain why the symptomatic effect of an $A_{2A}$ antagonist on its own appears modest compared with that of L-dopa (which can restore stimulation at all dopamine receptors). Evidence for this $A_{2A}$ receptor mechanism includes the correlation between the anti-parkinsonian effects of $A_{2A}$ antagonists and their ability to modulate GABA release and dopamine-dependent *c-fos* activation specifically in the indirect striatopallidal pathway [19,20].

## $A_{2A}$ antagonists as potential neuroprotectants in PD

Over past six years, converging epidemiological and experimental evidence has raised the exciting possibility that $A_{2A}$ receptor antagonism might protect dopaminergic neurons from degeneration in PD [13,21]. In 2000, Ross and colleagues reported an inverse relationship between the consumption of caffeine (1,3,7-trimethylxanthine), a non-specific adenosine receptor antagonist, and the risk of developing PD in 8004 Japanese–American men followed for 30 years as part of a prospective study [22]. The risk of PD adjusted for age and smoking was five times higher among men who reported no coffee consumption than among men who reported a daily consumption of 28 ounces of coffee or more. The finding was substantiated by a similar inverse relationship between the consumption of caffeinated (but not decaffeinated) coffee and the risk of developing PD in a larger and more ethnically diverse cohort of prospectively followed men [23]. Curiously, in women caffeine use is also linked to a reduced risk of PD but only among those who have not taken hormone-replacement therapy [24].

The neuroprotective potential of caffeine was further enhanced by genetic and pharmacological evidence that the $A_{2A}$ receptor can contribute to the degeneration of nigrostriatal dopaminergic neurons [13,21,25]. Caffeine at doses in mice corresponding to those of typical human exposure, which lead to blockade of adenosine $A_1$ in addition

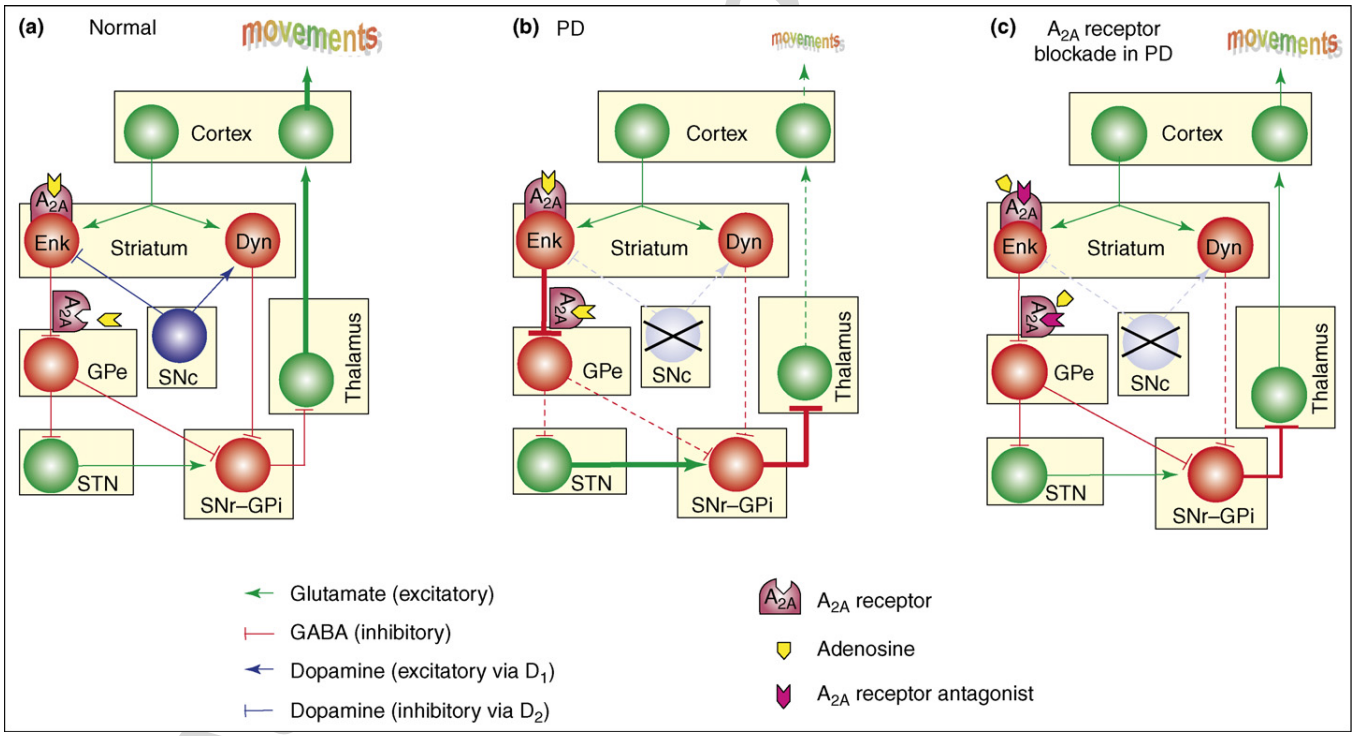


**Figure 2**. Proposed mechanism of symptomatic anti-parkinsonian activity of $A_{2A}$ receptor antagonists. The striatum is linked to the substantia nigra pars reticulata and globus pallidus pars interna complex (SNr–GPi) via direct (striatonigral) and indirect (striatal–pallidal–subthalamic–nigral) pathways. According to evolving models of basal ganglia organization and function [74], in the 'normal' state **(a)**, dopamine (blue) from neurons of the substantia nigra pars compacta (SNc) acts on stimulatory $D_1$ receptors of the direct striatonigral pathway and on inhibitory $D_2$ receptors of the indirect pathway to facilitate intricate and rapid movements. Adenosine, via $A_{2A}$ receptors on striatopallidal neurons in the striatum and globus pallidus pars externa (GPe), excites neurons in the indirect pathway, thus opposing the activation of $D_2$ receptors. **(b)** Degeneration of the SNc in Parkinson's disease (PD) removes dopamine input to the striatum. This disinhibits striatal spiny projection neurons of the indirect pathway, boosting their inhibitory GABA-mediated (red) influence on the GPe, which in turn leads to disinhibition of the excitatory glutamate-mediated (green) transmission of the subthalamic nucleus (STN). Depletion of dopamine also leads to decreased activation of striatal spiny neurons in the direct pathway. The resulting imbalance between the activity in the direct and the indirect pathways leads to markedly increased inhibitory output from the SNr–GPi complex. The subsequent excessive inhibition of thalamocortical neurons produces the characteristic reduction of movements of PD. **(c)** $A_{2A}$ receptor blockade in PD should relieve overactivity of striatopallidal and subsequently STN neurons, thereby restoring some balance between the direct and the indirect pathways. Note that an $A_{2A}$ antagonist together with a low dose of L-dopa (which elevates extracellular levels of dopamine that stimulates both $D_1$ and $D_2$ receptors) would facilitate the inhibitory cellular action of $D_2$ receptors on striatopallidal neurons. In turn, through the basal ganglia circuitry or collaterals from striatal medium-spiny neurons [75], this might indirectly facilitate activation of the striatonigral pathway stimulated by $D_1$ receptors [4] (not shown). Adapted, with permission, from Ref. [13] and based on references therein.

to $A_{2A}$ receptors in the brain, dose-dependently attenuates the loss of striatal dopaminergic terminals and nigral dopaminergic neurons that is triggered by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [13,25]. This protective effect of caffeine was also observed with several relatively selective antagonists of $A_{2A}$ (but not $A_1$) receptors, including KW-6002 [13,26]. Similarly, genetic deletion of the $A_{2A}$ receptor can attenuate the loss of striatal dopamine and dopaminergic terminals in the mouse MPTP model [25]. Interestingly, neuroprotection by caffeine, in contrast to its motor stimulant effect, does not exhibit tolerance after repeated prior injection [27]. Another striking feature of protection by caffeine against MPTP toxicity is its attenuation or absence in the presence of estrogen [28], closely paralleling the aforementioned relationship between caffeine and female hormone replacement in PD epidemiology. These complementary pharmacological and genetic studies provide compelling evidence that caffeine and more selective $A_{2A}$ receptor inactivation generally reduce the neurotoxic effects on dopaminergic neurons in animal models of PD neurodegeneration, although it should be noted that these laboratory models are as yet unproven as predictors of neuroprotection in humans. Thus the laboratory data suggest (but do not prove) that neuroprotection by caffeine is the basis for the reduced risk of developing PD among coffee drinkers and other caffeine consumers. It would be worthwhile to evaluate further whether the neuroprotective effect of $A_{2A}$ antagonism extends beyond acute neurotoxin models of PD and ultimately to PD patients.

The inferred pathophysiological role of endogenous adenosine acting through $A_{2A}$ receptors to hasten neurodegeneration is not restricted to dopaminergic neurons. $A_{2A}$-antagonist-mediated neuroprotection extends beyond PD models of nigrostriatal neuron degeneration. In fact, neuroprotection by $A_{2A}$ receptor antagonism was first reported in a global ischemia model [29] and further substantiated in other models of ischemic and excitotoxic brain injury in cortical regions [30,31]; this might also be of therapeutic relevance to $A_{2A}$ antagonist use in PD, because in its later stages cortical and basal ganglia neurodegeneration both contribute to the disease. In addition, $A_{2A}$ receptor antagonists can limit the damage to striatal output neurons that is induced by mitochondrial toxins [32–34], and can reduce formation of β-amyloid-induced aggregations in cultured cells [35], in models of both Huntington's disease and Alzheimer disease. However, $A_{2A}$ receptor inactivation has been found both to exacerbate and to alleviate the death of striatal neurons under different conditions, possibly owing to differential postsynaptic and presynaptic actions of the $A_{2A}$ receptor in striatum [32]. This suggests that any neuroprotective benefits of $A_{2A}$ antagonists in humans might apply to only a subset of degenerative CNS diseases.

The mechanism by which $A_{2A}$ receptor antagonists confer protection against death of dopaminergic neurons is not clear at present. However, the fact that their neuroprotective effects extend to other types of neurons in the basal ganglia and cerebral cortex supports a broad-based CNS mechanism that acts through common cellular elements – neuronal, glial and/or immunological. For example, $A_{2A}$ receptor activation on widely distributed glutamatergic nerve terminals or astrocytes might enhance glutamate release and thereby contribute to an 'excitotoxic' component of neuronal cell death [36–38]. Alternatively, $A_{2A}$ receptors on microglia and other immune cells might facilitate neuroinflammation that has an important role in brain injury [39,40], consistent with a recent report that neuroprotection against MPTP neurotoxicity by KW-6002 is associated with inhibition of microglial activation in the substantia nigra [41]. A better understanding of how the multiple actions of $A_{2A}$ receptors influence survival of dopaminergic neurons might further establish $A_{2A}$ antagonism as a potential neuroprotective strategy for the treatment of PD.

## $A_{2A}$ receptors in dyskinesias and non-motor targets

With the progressive loss of dopaminergic neurons in PD come other disabling motor and behavioral problems. Chronic intermittent therapy using L-dopa (or a dopamine agonist) can conspire with the hypodopaminergic state of PD to produce progressively briefer motor benefits and progressively more disruptive involuntary movements (dyskinesias) in response to each dose. The prevention and suppression of L-dopa-induced dyskinesias (LID) have become major targets of new non-dopamine approaches.

Although early clinical data suggest that $A_{2A}$ antagonists do not suppress LID after it is established [11,12], preclinical findings raise the possibility that $A_{2A}$ blockade can reduce the risk of developing LID in the first place. Hemi-parkinsonian rats that are treated daily with L-dopa develop a markedly sensitized rotational response over weeks. When an $A_{2A}$ antagonist was co-administered with a low dose of L-dopa, the acute effect of the combination matched that of a higher dose of L-dopa alone. By contrast, chronic use of the combined treatment caused no sensitization [9], supporting a rationale for combining $A_{2A}$ antagonists with a low dose of L-dopa earlier in the disease.

In addition to reducing the risk of LID by enabling use of a reduced dose of L-dopa, $A_{2A}$ antagonists might protect against LID more directly, by disrupting the pathophysiology of LID. $A_{2A}$ receptors are crucial in the development of a persistent sensitized motor response to L-dopa in parkinsonian mice, because their $A_{2A}$ knockout littermates did not develop this response [42]. Similarly, in parkinsonian rats repeatedly treated with L-dopa to model the motor complications of its chronic use in PD, pairing an $A_{2A}$ antagonist with L-dopa prevented shortening of the duration of the rotational response [43]. However, in another model, in which LID is represented by abnormal involuntary movements rather than enhanced rotational behaviors, $A_{2A}$ antagonism showed no attenuating effect on the development of these movements [44]. This highlights the uncertainty both over which rodent models of LID are most useful [45] and over the role of $A_{2A}$ receptors in LID. Nevertheless, using the gold-standard primate model of dyskinesias, Bibbiani *et al.* found that chronic treatment using the $A_{2A}$ antagonist KW-6002 completely prevented the induction of dyskinesias that is normally seen in parkinsonian (MPTP-lesioned) cynomolgus monkeys after 1–2 weeks of daily administration of a dopamine receptor agonist [43]. Most striking was their observation that continuing this treatment following cessation of adjunctive KW-6002 administration led to the induction of dyskinesias with

the same 1–2 week lag time, strengthening the rationale for a trial of early $A_{2A}$ antagonist treatment as an adjunct to L-dopa.

Although mechanisms of dyskinesia are poorly understood, the chronic administration of L-dopa has been clearly correlated with features of dyskinesia such as the long-term increase in striatal dynorphin mRNA, the modified phosphorylation state of NMDA and AMPA receptor subunits, and inhibition of activity in the substantia nigra pars reticulata [43,46–49]. By contrast, daily L-dopa administered in combination with an $A_{2A}$ antagonist or in the absence of $A_{2A}$ receptors did not trigger these modifications (and might attenuate the increase in enkephalin mRNA levels that is induced by 6-OHDA), providing possible mechanisms for the low dyskinetic potential observed with this drug combination [13,42,43,47,48].

Clinical development of $A_{2A}$ antagonists as anti-parkinsonian agents should also take into account evidence for $A_{2A}$ receptor involvement in common non-motor CNS disorders in PD patients, such a depression, psychosis, dementia and disrupted arousal and sleep states. For example, because $A_{2A}$ antagonists might have anti-depressant properties [50], it will be important to consider whether motor and subjective improvement reflects elevation of mood, which is commonly depressed in PD. Whereas an antidepressant side benefit could enhance their therapeutic impact, $A_{2A}$ antagonists also carry theoretical risks. For example, the possibility of a pro-psychotic side effect of $A_{2A}$ antagonists has been raised, given their demonstrated ability to enhance transmission by $D_2$ receptors and the well-known pro-psychotic effects of $D_2$ agonists. Lastly, the well-established peripheral effects of $A_{2A}$ receptors [51], particularly their anti-inflammatory actions, must also be addressed in the clinical development of $A_{2A}$ antagonists, especially in advanced PD patients who are at higher risk of and from infections.

## $A_{2A}$ antagonists for PD: lackluster or blockbuster?

This question may seem overly theatrical, with 'lackluster' too pessimistic given the positive results of initial clinical trials using $A_{2A}$ antagonists for PD, and 'blockbuster' too optimistic given that this label is conventionally reserved for conditions even more prevalent than PD. Nevertheless, the possibilities for $A_{2A}$ antagonism in PD are currently wide open. On the one hand, more than the initially demonstrated mild symptomatic benefit of adjunctive $A_{2A}$ antagonists will probably be needed to have a major effect on how we treat PD. On the other hand, $A_{2A}$ antagonists might offer disease-modifying and more substantial symptomatic benefits. So although the promise of these compounds is counterbalanced by the long odds inherent in clinical drug development, $A_{2A}$ antagonism clearly offers a uniquely hopeful and realistic opportunity for improving PD treatment.

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# Clinical trials for neuroprotection in Parkinson's disease: overcoming angst and futility?

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**Purpose of review**

To summarize recently published results of neuroprotection trials for Parkinson's disease, and discuss them in the context of evolving concepts in clinical study design and animal models.

**Recent findings**

Despite compelling preclinical evidence from laboratory models suggesting potential neuroprotective benefits, the antioxidant, antiapoptotic, antiexcitotoxic, immunomodulatory and neurotrophic agents studied to date have not shown clear benefit in human studies. The futility study design, an alternative approach focused on efficiently excluding less promising compounds, has been adopted recently to investigate four candidate neuroprotectants. A delayed-start trial design has also been introduced in a study of the monoamine oxidase inhibitor rasagiline, demonstrating a possible neuroprotective effect as well as its clear symptomatic benefit. In parallel with these clinical innovations, preclinical research initiatives are identifying new animal models that more closely resemble the clinical course and pathology of Parkinson's disease.

**Summary**

Angst over disappointing results of neuroprotection trials in Parkinson's disease has engendered efforts to refine animal models at one end of the therapeutics pipeline, and to optimize clinical trial design at the other. Building on new insights into the genetics, epidemiology and pathogenesis of Parkinson's disease, these recent improvements in 'translational infrastructure' will enhance the prospects of achieving the critical goal of slowing the progression of disability.

**Keywords**

clinical trial, futility study, neuroprotection, Parkinson's disease

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**Abbreviations**

| | |
|---|---|
| **6-OHDA** | 6-hydroxydopamine |
| **MPTP** | 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine |
| **SPECT** | single photon emission computed tomography |
| **UPDRS** | Unified Parkinson's Disease Rating Scale |


1350-7540

## Introduction

Parkinson's disease is characterized clinically by the cardinal features of resting tremor, rigidity, bradykinesia, and gait difficulty with postural instability. At least in the early stages most of the motor dysfunction results from the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta. Highly effective symptomatic treatments, primarily based on dopamine replacement strategies, are available to treat early to moderate stage Parkinson's disease, providing significant improvement in motor function and quality of life. Despite advances in understanding of the pathogenesis of Parkinson's disease, however, efforts to identify a therapy to slow or reverse disease progression have been disappointing. Indeed, a recent practice parameter submitted by the American Academy of Neurology concluded that 'no treatment has been shown to be neuroprotective' [1••]. Thus, clinical and basic research focused on identifying these neuroprotective strategies addresses a major unmet need [2•].

The etiology of idiopathic Parkinson's disease remains uncertain. A number of genetic mutations have recently been identified in some patients with a familial form of the disorder (reviewed elsewhere in this volume). These mutations, however, account for only a small percentage of cases of Parkinson's disease. In addition, twin studies suggest that while genetic factors may predispose young-onset patients to Parkinson's disease, they appear to be less important in the majority of individuals with later onset disease [3]. Indeed, current models propose a complex interplay of biochemical and cellular processes, including oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammation, trophic factor deficiency, and apoptosis [4,5]. In light of the significant clinical impact that would be achieved by identifying agents slowing disease progression, the Committee to Identify Neuroprotective Agents in Parkinson's (CINAPS) was formed and assigned the task of prioritizing prospective neuroprotective candidates for clinical trials [6]. Four

**Table 1 Putative neuroprotective agents identified by the Committee to Identify Neuroprotective Agents in Parkinson's (CINAPS) group**

| Agent | Primary mechanism |
|---|---|
| Caffeine | Adenosine antagonist |
| Coenzyme $Q_{10}$ | Antioxidant/mitochondrial stabilizer |
| Creatine | Antioxidant/mitochondrial stabilizer |
| Estrogen | Unknown |
| GM-1 ganglioside | Trophic factor |
| Minocycline | Antiinflammatory/antiapoptotic |
| Nicotine | Unknown |
| GPI-1485 | Trophic factor |
| Pramipexole | Antioxidant |
| Rasagiline | Antioxidant/antiapoptotic |
| Ropinirole | Antioxidant |
| Selegiline | Antioxidant/antiapoptotic |

Data from [6].

primary criteria were used: scientific rationale, evidence of blood–brain barrier penetration, adequate safety and tolerability data, and efficacy in animal models and preliminary human studies. Based on these criteria, 12 compounds were selected as attractive candidates for further study (Table 1). Here, we review the recent clinical trials focused on these and other agents, and discuss the limitations of current clinical study design and preclinical animal models.

## Lessons from early neuroprotective trials

Clinical neuroprotection trials for Parkinson's disease date back to the early 1980s, when oxidative stress and oxidative activation of putative environmental protoxins were first proposed as mechanisms for dopamine neuron loss. On the basis of this hypothesis, early trials focused on the potential effectiveness of selegiline, a selective, irreversible inhibitor of monoamine oxidase type B (MAO-B) that had been shown to protect against dopamine cell loss in animal models (Table 2) [7–11], reviewed in [29••]. Using defined clinical endpoints [time until symptomatic treatment was required, or changes in the Unified Parkinson's Disease Rating Scale (UPDRS)], these studies demonstrated a clear benefit in those who received selegiline. It became evident, however, that these results could similarly be explained by a symptomatic effect of the study intervention.

In order to overcome the confounding effects of symptomatic benefit, several studies employed radiotracer imaging as a surrogate measure of dopaminergic nigrostriatal neuron integrity. $^{123}$I-β-CIT single photon emission computed tomography (SPECT) reflects binding to the dopamine transporter on dopaminergic cells, and [$^{18}$F]fluorodopa PET measures the uptake and conversion of the tracer into fluorodopamine in surviving neurons. This approach was used to study the potential neuroprotective effects of the nonergot dopamine agonists pramipexole and ropinirole [20,21]. Both of these agents (compared with levodopa) resulted in less reduction in uptake of the putative biomarker over the course of the treatment period, suggesting a possible neuroprotective effect with agonist therapy. The ELLDOPA study [22], investigating the effects of levodopa on disease progression, has brought the reliability of imaging measures into question. In this study, patients treated with carbidopa-levodopa showed a smaller decrease in UPDRS scores, but a significantly greater decline in β-CIT uptake. Thus, as radiotracer imaging does not consistently correlate with clinical endpoints, it has not been established as an appropriate surrogate of neurodegeneration in neuroprotection trials for Parkinson's disease [30].

## Antioxidants

The focus on oxidative stress as a mechanism of dopaminergic cell loss has led to several additional trials examining whether antioxidant therapy can modify the course of Parkinson's disease. Coenzyme $Q_{10}$ is a cofactor in the electron transport chain, and has potent antioxidant effects. It is also thought to stabilize mitochondrial function. In a pilot study of coenzyme $Q_{10}$ in early Parkinson's disease, a total of 80 patients were assigned to placebo or one of three different doses (300, 600 and 1200 mg per day) [16]. Using change in UPDRS as the primary outcome measure, there appeared to be an inverse trend between dose and degree of clinical change, suggesting possible neuroprotection. The potential benefit of coenzyme $Q_{10}$ was most clearly suggested at the highest dose, though at this and other doses there was no significant effect on secondary measures of a timed tapping test of motor function or of the time until levodopa therapy initiation was warranted. Since that study, daily doses as high as 2400 mg have been shown to be well tolerated [31].

Creatine is a dietary supplement that plays an important role in mitochondrial ATP production. In animal models of Parkinson's disease, oral creatine supplementation has been shown to have a neuroprotective effect [32]. The results from a 2-year placebo-controlled pilot efficacy trial were recently reported [18•]. Changes in dopamine transporter SPECT were used as the primary outcome measure, and the UPDRS and 36-item Short Form Health Survey (SF-36) were secondary outcome measures for disease progression. In this study, creatine supplementation had no significant effect on SPECT variables, nor did it result in a change in overall UPDRS scores. With creatine, there was a slight improvement in mood (as assessed by UPDRS subscale I, 'Mentation, behavior, mood'). There was also a lower relative increase in agonist dose in patients treated with creatine, although this reflected primarily a difference in baseline dose rather than final dose. The authors concluded, however, that the negative results could potentially be related to the low doses used in the study.

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**Table 2 Published randomized neuroprotection trials in Parkinson's disease**

| Class | Agent | *n* | Primary outcome | Duration | Comments | Reference |
|---|---|---|---|---|---|---|
| Antioxidant/MAO inhibitor | Selegiline | 800 | Time to symptomatic treatment | | DATATOP | [7] |
| | Selegiline | 54 | Time to symptomatic treatment | 3 years | | [8] |
| | Selegiline | 157 | Time to symptomatic treatment | 1–3 years | | [9] |
| | Selegiline | 101 | Change in UPDRS | 14 months | 2 month washout | [10] |
| | Selegiline | 79 | Change in UPDRS | 60 months | 1 month washout | [11] |
| | Lazabemide | 321 | Time to symptomatic treatment | 12 months | | [12] |
| | Rasagiline | 404 | Change in UPDRS | 12 months | Delayed start | [13] |
| | Selegiline | 157 | Change in UPDRS | 7 years | With levodopa | [14] |
| Antioxidant | Vitamin E | 800 | Time to symptomatic treatment | | DATATOP | [15] |
| | Coenzyme Q10 | 80 | Change in UPDRS | 16 months | QE2 | [16] |
| | Coenzyme Q10 | 213 | Change in UPDRS | 12 months | Futility study | [17••] |
| | Creatine | 60 | [$^{123}$I]-FP-CIT SPECT | 24 months | | [18•] |
| | Creatine | 200 | Change in UPDRS | 12 months | Futility study | [19•] |
| DA replacement | Pramipexole | 82 | [$^{123}$I]-β-CIT SPECT | 46 months | CALM-PD | [20] |
| | Ropinirole | 186 | [$^{18}$F]-dopa PET | 24 months | REAL-PET | [21] |
| | Levodopa | 360 | Change in UPDRS | 40 weeks | ELLDOPA; 2 week washout | [22] |
| Glutamate antagonist | Riluzole | 20 | Change in UPDRS | 6 months | | [23] |
| Trophic factor | GDNF | 50 | Change in UPDRS motor score | 8 months | | [24] |
| | GDNF | 34 | Change in UPDRS motor score | 6 months | | [25] |
| Neuroimmunophilin ligand | GPI-1485 | 300 | Change in UPDRS motor score | 6 months | | Discussed in [26] |
| | GPI-1485 | 213 | Time to symptomatic treatment | 12 months | Futility study | [17••] |
| Antiapoptotic agent | TCH346 | 301 | Change in UPDRS | 12–18 months | | [27••] |
| | CEP-1347 | 806 | Change in UPDRS | Average 21.4 months | PRECEPT | [28] |
| | Minocycline | 200 | Time to symptomatic treatment | 12 months | Futility study | [19•] |

MAO, monoamine oxidase; UPDRS, Unified Parkinson's Disease Rating Scale; SPECT, single photon emission computed tomography; DA, dopamine; GDNF, glial cell line-derived neurotrophic factor.

As a step to assessing whether coenzyme $Q_{10}$ or creatine are suitable for further study as neuroprotective agents, phase II studies were conducted, concluding that neither agent could be rejected as futile (see below) [17••,19•]. On the basis of these data, larger multicenter studies using coenzyme $Q_{10}$ and creatine are being pursued.

## Antiapoptotic agents

Apoptosis, or programmed cell death, has been proposed to contribute to the pathogenesis of neurodegenerative disorders, including Parkinson's disease, although the neuropathologic evidence remains somewhat controversial [33]. Despite this uncertainty, there are numerous reports to suggest that interfering with apoptotic pathways can protect against neuronal cell death [34–36]. One class of antiapoptotic agents, the propargylamines, has been proposed as neuroprotective agents [37•]. TCH346 (*N*-methyl-*N*-propargyl-10-aminomethyl-dibenzo[b,f]-oxepin; also called CGP3466) is a propargylamine that resembles structurally selegiline, but lacks MAO inhibitor activity. Its antiapoptotic mechanism of action is thought to involve binding to glyceraldehyde-3-phosphate dehydrogenase [38]. In animal models of Parkinson's disease, the agent has been shown to protect against neurodegeneration and associated behavioral deficits [39,40]. A recent clinical study examined the putative neuroprotective effects of TCH346 on disease progression [27••]. Patients were randomly assigned to placebo or one of three treatment doses. After a 12–18-month treatment period and 4-week washout, they failed to show any difference in the primary endpoint (time to disability requiring dopaminergic therapy) or secondary endpoints (change in UPDRS or the Parkinson's disease questionnaire (PDQ)-39, a measure of quality of life).

CEP-1347 inhibits the mixed lineage kinases (MLKs) that activate the c-Jun N-terminal kinase (JNK) signaling cascade, a pathway that mediates apoptotic cell death. MLK inhibition and disruption of this pathway have consistently been shown in preclinical models to enhance neuronal survival [41,42]. These promising data prompted the PRECEPT trial, the largest neuroprotection trial completed to date, in which patients not yet requiring dopaminergic therapy were randomized to placebo or one of the doses of CEP-1347 [28]. Using time to disability requiring dopaminergic therapy as the primary endpoint, and changes in UPDRS and β-CIT SPECT imaging as secondary endpoints, this study was concluded early (average of 21.4 months of follow up) after an interim analysis showed that a greater percentage of patients on the lowest and highest doses of experimental drug (10 and 50 mg twice a day) reached the primary endpoint, compared with the placebo group. Similarly, all active treatment groups showed a greater decline in striatal β-CIT. Thus, CEP-1347 was shown to be ineffective at modifying disease progression in Parkinson's disease.

Based on these negative clinical trials, the relevance of apoptosis to designing neuroprotective agents requires reassessment [43••]. Alternatively, it has been suggested that apoptosis may contribute as just one of several routes to neuronal degeneration, such that blockade of apoptosis may lead to a different (e.g. necrotic) cell death pathway that may produce at least as much neuronal injury [44,45].

## The 'delayed start' design

Recently, the use of a 'delayed start' design has been proposed for neuroprotection trials [13,46••]. As noted, symptomatic effects of study interventions can confound interpretation when clinical measures are used as surrogates of disease progression. While this is addressed in part by incorporation of a 'washout' phase, when clinical performance is compared between untreated baseline and final visits, it is difficult to exclude the possibility that the treatment may have long-lasting effects that exceed the duration of the washout. Ethical issues have also been raised about withholding effective treatment during the washout period.

In the delayed start design, some study participants begin treatment with the experimental agent immediately, while others begin after a delay. This design presumes that the symptomatic benefit of the medication is similar in both groups at the end of treatment. Thus, any change in outcome measure should reflect a disease-modifying effect. The delayed start design has been used to study the effects of rasagiline, a propargylamine MAO-B inhibitor [13] that has been shown to protect against 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic cell loss in animal models [47]. In an initial 6-month placebo-controlled phase, treatment with rasagiline resulted in improvement in Parkinsonian symptoms [48]. Following this phase, study participants previously treated with placebo were given rasagiline (2 mg per day) and compared with individuals taking rasagiline from the start. Those treated with rasagiline (1 or 2 mg daily) for 12 months showed a lesser decline in clinical performance (as measured by change in UPDRS scores) than those only taking the medication for 6 months. This study has been interpreted to suggest that rasagiline can slow the rate of disease progression, prompting a similar but longer and larger study now underway [49].

## The use of futility studies

The failure of agents proven effective in preclinical studies to have any efficacy in human studies has led clinical investigators to ask whether there is a more efficient way to screen out compounds that are ineffective as neuroprotectants. In the last several years, a futility study design has been used to assess several prospective neuroprotective agents [50,51••,52•]. This methodology has been used previously to evaluate cancer treatments and more recently, stroke treatments. Phase II pilot studies with such a design require a smaller number of patients over a shorter time period than large phase III efficacy studies. They focus on distinguishing between potentially effective agents appropriate for larger randomized studies, and agents that are demonstrably 'futile' (at least at the dose tested). In order for such an approach to be effective, it is necessary to identify clinical outcome measures that would be expected to change sufficiently in untreated patients over 6–12 months, the short time period intended in futility studies. An analysis of historical Parkinson's disease data sets identified change in total UPDRS or change in motor plus activities of daily living (ADL) UPDRS (subscales 2 and 3) as requiring the smallest sample sizes [50]. While some efficacy data can be obtained from such studies, however, they lack (by design) sufficient statistical power to test whether a drug is actually disease modifying.

A randomized, double-blind futility trial was conducted by the National Institutes of Health (NIH) Exploratory Trials in Parkinson's disease (NET-PD) program, examining a potential neuroprotective effect of creatine and minocycline [19•]. Minocycline is a semi-synthetic second-generation tetracycline that has been proposed to protect against MPTP-induced dopaminergic cell loss via its antiinflammatory action [53]. A total of 200 participants were randomized to receive either placebo, creatine (10 g per day), or minocycline (200 mg per day). Using change in total UPDRS as the primary endpoint, neither agent could be rejected as futile. All three treatment groups were fairly well tolerated (91% in creatine group, 77% in minocycline group); the most common adverse events included upper respiratory symptoms (26%), joint pain (19%), and nausea (17%).

Neuroimmunophilins are a family of proteins present in the nervous system that were initially identified as targets of the immunosuppressive agents FK506 and rapamycin [26,54]. The neuroimmunophilin ligand GPI-1046 has been shown to reverse the loss of corticostriatal long-term potentiation in 6-hydroxydopamine lesioned rodents, suggesting that it may protect dopaminergic cell function [55]. An initial study using a similar agent, GPI-1485, at a maximal dose of 4000 mg daily showed no treatment benefit on the primary outcome of change in UPDRS motor score [26]. To assess whether GPI-1485 deserves further study, the agent was tested along with coenzyme $Q_{10}$ using the futility trial design [17••]. In this study, a total of 213 individuals were randomized to placebo, coenzyme $Q_{10}$ (2400 mg per day), or GPI-1485 (4000 mg per day). Over a 12-month trial period (or until symptomatic therapy was warranted), the primary outcome measure (change in total UPDRS scores) again did not meet the prespecified criteria for futility of either agent. Like the other two agents studied, the study

interventions were well tolerated, with a similar set of adverse effects.

Based on these studies, all four agents tested remain potential candidates for larger phase III trials. Several important issues, however, have been raised by these reports. The placebo group in the creatine/minocycline study showed a mean change in total UPDRS of 8.39, whereas the coenzyme $Q_{10}$/GPI-1485 placebo group showed a much smaller change (6.31). When the four treatment arms were analyzed in comparison to the combined mean of both placebo groups and the placebo arm of the PRECEPT trial, only creatine was not found to be futile (i.e. potentially disease modifying). Thus, the study conclusion depends on the placebo data used to determine the futility threshold. This raises the concern that changes in UPDRS, the primary response variable used in these trials, may be an inadequate primary outcome measure. It may be necessary to employ multiple outcome measures or global statistical tests [46••].

## Limitation of preclinical rationales: animal models

The recent negative results from clinical trials of a small but diverse set of potential neuroprotectants may reflect a series of individual inadequacies in the identification of candidate drugs or in the design and execution of clinical trials for neuroprotection in Parkinson's disease. Alternatively, they could reflect a broader systematic problem in our identification or testing of candidate agents. Accordingly, in addition to clinical trial design, the adequacy of available animal models has also come into question. Short of being confirmed as predictive of a drug's neuroprotective potential in humans, an animal model of neurodegeneration can be validated by demonstrating that it shares the progressive nature of the disease and the defining pathologic features. Most preclinical studies have used the MPTP or 6-hydroxydopamine (6-OHDA) model to characterize protective effects on dopaminergic cell loss [56]. Indeed, results from these models served as one of the primary evaluation criteria for inclusion in the list of attractive candidates by CINAPS [6]. MPTP is a mitochondrial complex I inhibitor, damaging dopamine neurons in part through the generation of reactive oxygen species. 6-OHDA, when administered intracerebrally, also induces oxidative injury by entering cells through catecholamine transporters. Nevertheless, these models have limitations [57]. The 6-OHDA model causes acute degeneration of nigrostriatal neurons, and does not cause the pathognomonic cytoplasmic inclusions (Lewy bodies) seen in Parkinson's disease. Similarly, most MPTP protocols use acute treatments and fail to produce Lewy bodies, though Lewy body-like cytoplasmic inclusions have been reported in a chronic MPTP infusion model [58]. Moreover, the effects of putative neuroprotectants have been shown to vary depending on the toxin administration protocol [59•]. In an effort to enhance the environmental relevance of toxin models of Parkinson's disease, the pesticide rotenone and herbicide paraquat have been used in toxin models of Parkinson's disease. While some reports indicate a chronic, progressive loss of nigrostriatal neurons, inconsistent pathologic effects have undermined their utility for testing neuroprotective compounds [60].

Recent genetic data have implicated dysfunction in the ubiquitin–proteasome system in the pathogenesis of Parkinson's disease (reviewed elsewhere in this volume). This led McNaught and colleagues [61,62•] to investigate the effect of systemic exposure to proteasome inhibitors. They reported that rodents treated with epoxomicin or PSI, two different proteasome inhibitors, for 2 weeks produced a progressive dopamine-responsive neurologic syndrome suggestive of Parkinsonism after a latency of 1–2 weeks. Moreover, pathologic analysis confirmed depletion of striatal dopamine and dopaminergic cell loss. If validated, such a model may be appropriate for testing putative neuroprotective compounds. Unfortunately, these findings have been difficult to confirm. A series of recent reports have highlighted the controversy, with a number of laboratories only partially replicating or completely failing to replicate the model (reviewed in [63••]) [64•–68•]. In light of this uncertainty, the proteasomal inhibition model, despite its early promise, requires further validation before it can be used to screen compounds for potential clinical use.

## Conclusion

Angst over the disappointing results from clinical neuroprotection trials for Parkinson's disease has engendered efforts to optimize neuroprotection trial design, as well as to refine the animal models in which candidate neuroprotectants are advanced. The early use of clinical measures such as time until dopaminergic therapy or change in clinical rating scales as primary outcomes were complicated by confounding effects of symptomatic improvement. While the use of delayed start designs may help to overcome these confounders, there is nevertheless a need to develop better, more objective biomarkers of disease progression. Futility studies may prove to be helpful to exclude more efficiently compounds that are unlikely to be useful. The variability in the calibration placebo in the first two futility trials, however, raises concern that compounds may be deemed futile or not futile inappropriately. In reexamining the process by which promising neuroprotectants are identified, the validity of our toxin-based animal models has been questioned, prompting new models that more closely mimic the progressive features and pathology of the disease. Continued advances in our understanding of genetic and environmental factors contributing to Parkinson's disease will lead to more promising

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therapeutic candidates. The prospects for their translation into clinical practice will be enhanced by further improvements in animal models and clinical trial designs for Parkinson's disease.

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ORIGINAL CONTRIBUTION

# Urate as a Predictor of the Rate of Clinical Decline in Parkinson Disease

ARCHIVES EXPRESS

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**Background:** The risk of Parkinson disease (PD) and its rate of progression may decline with increasing concentration of blood urate, a major antioxidant.

**Objective:** To determine whether serum and cerebrospinal fluid concentrations of urate predict clinical progression in patients with PD.

**Design, Setting, and Participants:** Eight hundred subjects with early PD enrolled in the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trial. The pretreatment urate concentration was measured in serum for 774 subjects and in cerebrospinal fluid for 713 subjects.

**Main Outcome Measures:** Treatment-, age-, and sex-adjusted hazard ratios (HRs) for clinical disability requiring levodopa therapy, the prespecified primary end point of the original DATATOP trial.

**Results:** The HR of progressing to the primary end point decreased with increasing serum urate concentrations (HR for highest vs lowest quintile = 0.64; 95% confidence interval [CI], 0.44-0.94; HR for a 1-SD increase = 0.82; 95% CI, 0.73-0.93). In analyses stratified by α-tocopherol treatment (2000 IU/d), a decrease in the HR for the primary end point was seen only among subjects not treated with α-tocopherol (HR for a 1-SD increase = 0.75; 95% CI, 0.62-0.89; vs HR for those treated = 0.90; 95% CI, 0.75-1.08). Results were similar for the rate of change in the Unified Parkinson's Disease Rating Scale score. Cerebrospinal fluid urate concentration was also inversely related to both the primary end point (HR for highest vs lowest quintile = 0.65; 95% CI, 0.44-0.96; HR for a 1-SD increase = 0.89; 95% CI, 0.79-1.02) and the rate of change in the Unified Parkinson's Disease Rating Scale score. As with serum urate concentration, these associations were present only among subjects not treated with α-tocopherol.

**Conclusions:** Higher serum and cerebrospinal fluid urate concentrations at baseline were associated with slower rates of clinical decline. The findings strengthen the link between urate concentration and PD and the rationale for considering central nervous system urate concentration elevation as a potential strategy to slow PD progression.

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IN HUMANS, URATE IS A MAJOR ANtioxidant as well as the end product of purine metabolism.[1,2] Its high concentrations in cerebrospinal fluid (CSF) and blood have been attributed to a mutation in the urate oxidase gene occurring late in hominid evolution.[3] Oxidative damage is suspected to contribute to the neurodegenerative process in Parkinson disease (PD),[4,5] and antioxidants like urate may provide an endogenous defense against the development and progression of PD.

Prospective epidemiological studies have demonstrated that healthy individuals with higher blood urate concentrations are at reduced risk for developing PD.[6-9] Similarly, a lower risk of PD has also been reported among individuals consuming diets that increase serum urate concentration[10] and among those with a history of gout.[11,12] Recently, we found that higher urate blood concentrations in patients in the early stages of PD predict a slower rate of disease progression, assessed by both clinical and neuroimaging measures.[13] These studies suggest that urate concentration measured systemically may serve as a robust predictor of the brain neurodegeneration that leads to the initiation and progression of PD.

The studies also raise the possibility that central nervous system urate directly protects against the neuronal degeneration underlying clinical deterioration in PD. Cerebrospinal fluid may more closely reflect

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the microenvironment of degenerating neurons than does blood.[14] Accordingly, we used the clinical database from a completed multicenter, randomized, placebo-controlled trial (the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism [DATATOP] trial)[15,16] to test the hypothesis that higher urate concentrations in both CSF and blood specimens from patients with PD predict a slower rate of clinical disease progression.

## METHODS

### STUDY DESIGN

The DATATOP study was a 2-year, double-blind, randomized trial originally designed to test the hypothesis that long-term treatment of early PD with the monoamine oxidase type B inhibitor deprenyl (selegiline hydrochloride) and/or the antioxidant α-tocopherol would extend the time until the emergence of disability requiring therapy with levodopa.[15] The 800 participants were enrolled between September 1987 and November 1988 at 28 sites across the United States and Canada.

### STUDY POPULATION

Subjects enrolled in the study had typical and early PD (Hoehn and Yahr stages 1 and 2) of less than 5 years' duration and were excluded if they used symptomatic PD medication or had severe tremor, serious dementia (Mini-Mental State Examination score ≤22), or depression (Hamilton Scale for Depression score ≥16). Subjects were reviewed and examined by neurologists who were PD specialists. After baseline evaluation, study participants were randomized according to a 2×2 factorial design to 1 of 4 treatment assignments: deprenyl (10 mg/d) and α-tocopherol placebo, α-tocopherol (2000 IU/d) and deprenyl placebo, active deprenyl and active α-tocopherol, or double placebo.[17]

### SERUM AND CSF URATE CONCENTRATIONS AND COVARIATES

Urate concentration was measured in serum samples collected at the baseline visit prior to treatment assignment. Serum was shipped without freezing to a central commercial clinical laboratory (SciCor, Indianapolis, Indiana) for immediate enzymatic assay of urate concentrations, which were available for 774 of the 800 enrolled subjects. Values maintained in a digitized database were not analyzed with respect to disease progression outcome measures until their retrieval in May 2006 specifically for this purpose.

Cerebrospinal fluid was collected at baseline after overnight bed rest from 730 subjects (ie, 91.2% of enrollees, with technical difficulties in performing lumbar punctures precluding the collection from the others)[18] and at the end of the study in 486 subjects. Specimens were rapidly frozen for storage at −70°C after first splitting all CSF collection tubes into aliquots with or without metabisulfite preservative added.[18] Baseline and final CSF urate concentrations were measured in 1991 by high-performance liquid chromatography with electrochemical detection from collection tubes containing the 18th to 20th milliliter of lumbar CSF flow in 2 selected subsets totaling 290 subjects who had provided both baseline and final CSF collections.[19] The values of CSF urate concentrations at baseline correlated well with those at the end of treatment or follow-up in both subsets (Spearman coefficient=0.69; $P<.001$), a result that supports the reproducibility of the assay as well as relatively stable within-person CSF urate concentrations. For the present analyses, in 2008 we obtained CSF aliquots from the same collection tubes (containing no metabisulfite preservative) and repeated the measurement of urate concentrations by high-performance liquid chromatography with electrochemical detection. For these assays, 50μM α-methyldopa served as an internal standard. Baseline CSF urate concentrations could be determined in 713 participants (ie, 97.7% of those from whom a baseline CSF sample was obtained and stored). Although mean CSF urate concentrations were lower than those measured in 1991, a good correlation was found between original urate concentrations and those measured in 2008 among the 277 individuals in both sets (Spearman coefficient=0.72; $P<.001$). Furthermore, baseline serum urate concentrations correlated more strongly with baseline CSF urate concentrations measured in 2008 ($r=0.73$) than in 1991 ($r=0.58$). These results provide evidence of the stability of urate in these samples and of the accuracy of CSF urate concentration measurements.

### CLINICAL EVALUATION AND OUTCOMES

Following the baseline visit and initiation of study drugs, subjects were scheduled for visits every 3 months until 24 months had elapsed.[17] At each visit the site investigator evaluated the subject for disability sufficient to require dopaminergic therapy, the primary end point for the study, and for the secondary response variables, including the Unified Parkinson's Disease Rating Scale (UPDRS) score (sum of the motor, cognitive, and activity of daily living subscale scores).[17] Because the UPDRS score is modified by the dopaminergic treatment instituted at the primary end point, the annualized rate of change in the UPDRS score was determined based on change from baseline to the primary end point (or the final visit if the primary end point was not reached) for each subject and was calculated as follows: [(total UPDRS score at the last assessment before initiation of dopaminergic treatment − total UPDRS score at baseline)/number of days between the 2 assessments] × 365 d/y. The vital status and date of death of participants in the DATATOP trial were collected in 2001 to 2002 as previously described.[20] The shortest time elapsed between enrollment and vital status update was 13 years. Information was available for 768 subjects with baseline serum urate concentration measurement.

### STATISTICAL ANALYSIS

In the original trial, the hazard ratios (HRs) for the primary end point were 0.50 (95% confidence interval [CI], 0.41-0.62) among patients assigned to deprenyl and 0.91 (95% CI, 0.74-1.12) among patients assigned to α-tocopherol.[17] Accordingly, all of the analyses were adjusted for assignment to deprenyl vs placebo.

Cox proportional hazards models were used to estimate the HRs of reaching the primary end point according to quintiles of baseline serum urate concentration, adjusting for sex, age (in 5-year groups), and treatment assignment (deprenyl vs placebo). Initial analyses were conducted using quintiles based on the combined urate concentration distribution in men and women. However, because this categorization resulted in a markedly skewed distribution within sex as expected, we also conducted analyses based on sex-specific quintiles. Tests for trend were conducted by including serum urate concentration as a continuous variable in the proportional hazards models. Potential confounding was assessed by adjusting the regression analyses for body mass index (BMI) (calculated as weight in kilograms divided by height in meters squared) and use of antihypertensive drugs or nonsteroidal anti-inflammatory drugs (use vs no use). With the exception of BMI, these adjustments did not affect the results. Therefore, only the treatment-, age-, and sex-adjusted results or the treatment-, age-, sex-, and BMI-

**Table 1. Baseline Characteristics of Study Participants According to Quintiles of Baseline Serum Urate Concentration**

| Characteristic | Baseline Serum Urate Concentration Quintile | | | | | All Subjects |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Serum urate concentration, mg/dL | ≤3.90 | 3.91-4.60 | 4.61-5.20 | 5.21-6.20 | ≥6.21 | 5.1[a] |
| Subjects, No. | 162 | 140 | 149 | 165 | 158 | 774 |
| Female, % | 75.9 | 40.7 | 24.2 | 17.0 | 12.7 | 34.1 |
| Age, median, y | 62 | 63 | 61 | 63 | 63 | 62 |
| BMI, mean | 23.7 | 26.2 | 26.1 | 27.1 | 28.4 | 26.3 |
| Current smokers, % | 9.3 | 5.7 | 12.1 | 10.3 | 6.3 | 8.8 |
| Using antihypertensive drugs for hypertension, % | 6.8 | 7.1 | 7.4 | 8.5 | 15.7 | 9.2 |
| Using thiazides, % | 2.5 | 2.9 | 3.4 | 4.2 | 6.9 | 4.0 |
| Using NSAIDs, % | 22.2 | 22.1 | 20.1 | 27.3 | 17.6 | 21.9 |
| Cardiac comorbidity, % | 17.9 | 25.0 | 24.8 | 27.9 | 36.5 | 26.5 |
| Time since onset per rater, y | 1.9 | 2.0 | 2.0 | 2.0 | 1.9 | 2.0 |
| Total UPDRS score, mean | 25.1 | 25.7 | 25.4 | 26.4 | 24.5 | 25.5 |
| UPDRS rest tremor score, mean | 4.5 | 4.8 | 4.4 | 4.7 | 4.6 | 4.6 |
| MMSE score, mean | 28.8 | 28.9 | 29.0 | 28.8 | 28.8 | 28.9 |

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); MMSE, Mini-Mental State Examination; NSAIDS, nonsteroidal anti-inflammatory drugs; UPDRS, Unified Parkinson's Disease Rating Scale.
[a]Value is expressed as the mean.

adjusted results are presented. Possible interactions were explored by including the cross-product of serum urate concentration (continuous variable) with age (continuous in years), sex, or deprenyl and α-tocopherol treatments in the proportional hazards model. No interaction terms were significant, and only results that do not include these terms are reported. The results of these exploratory analyses, however, suggested a possible interaction between α-tocopherol treatment and serum urate concentration. Because both α-tocopherol and urate have antioxidant properties, this interaction has some biological plausibility. This interaction was examined further by estimating the HRs for the primary outcome in groups of subjects classified according to both their serum urate concentration and treatment group. The relationship between serum urate concentration and rate of change in the UPDRS score (between baseline and the last visit before reaching the primary end point) was assessed by linear regression with adjustment for treatment, age, and sex using both common quintiles of serum urate concentration and sex-specific quintiles. This analysis was complemented by a repeated-measures analysis using all available UPDRS score determinations. This analysis was conducted by fitting a linear mixed model with random intercept and slope and fixed effects for treatment, age, sex, urate concentration, and the interaction between urate concentration and α-tocopherol treatment. The association between serum urate concentration and time from study enrollment until death was investigated using Cox proportional hazards models adjusted for treatment, age, sex, and smoking history (pack-years), with or without further adjustment for cardiac morbidity at baseline. Analyses for CSF urate concentration were conducted in the same manner.

## RESULTS

### SERUM URATE CONCENTRATION

Serum urate concentration at baseline was available for 774 of the 800 subjects (96.8%; 510 men and 264 women) enrolled in the trial. Selected characteristics of these subjects are shown in **Table 1**. As expected, serum urate concentrations correlated positively with being male, BMI, use of thiazide diuretics, and hypertension. Use of calcium channel blockers was reported by only 17 patients and showed no relationship to serum urate concentration.

Overall, 369 (47.7%) of these participants progressed to disability sufficient to require levodopa therapy during follow-up. The HR of reaching this primary end point declined with increasing concentrations of serum urate ($P$ for trend = .002) and was 36% lower among subjects in the top quintile as compared with those in the bottom quintile of serum urate concentration (HR = 0.64; 95% CI, 0.44-0.94) (**Table 2**). This association was stronger in men than in women, although a test for interaction of urate concentration with sex was not significant ($P$ = .54). Further, in both sexes, the HR for reaching the primary end point decreased with increasing BMI ($P$ for trend = .05 in men, $P$ for trend = .02 in women). After adjustment for BMI, the association between serum urate concentration and the primary clinical end point was partially attenuated; the HRs for a 1-SD increase in serum urate concentration were 0.89 in all subjects ($P$ = .07), 0.85 in men ($P$ = .04), and 1.01 in women ($P$ = .94).

When subjects were classified simultaneously according to serum urate concentration and α-tocopherol treatment, a decreasing HR for reaching the primary end point with increasing serum urate concentration was observed among untreated subjects (HR = 0.75; 95% CI, 0.62-0.89; $P$ = .001) but not among those treated (HR = 0.90; 95% CI, 0.75-1.08; $P$ = .24), consistent with comparisons of baseline urate concentration quintiles (**Figure 1**A) and unadjusted Kaplan-Meier analyses (eFigure; http://www.archneurol.com) in subgroups without or with α-tocopherol treatment. Conversely, randomization to α-tocopherol treatment appeared to lower the HR of reaching the primary end point among subjects in the lowest quintile of serum urate concentration (HR = 0.59; 95% CI, 0.36-0.97) but not among those with a higher serum urate concentration (Figure 1A). Further analyses were conducted within sex. In men, the HRs for a 1-SD increase in serum urate concentration were 0.74 (95% CI, 0.59-0.92; $P$ = .008) among subjects not receiving α-tocopherol and

**Table 2. Hazard Ratios for Reaching the Primary End Point According to Common Quintiles of Baseline Serum Urate Concentration or Corresponding to a 1-SD Increase in Serum Urate Concentration[a]**

| Serum Urate Concentration Quintile | Serum Urate Concentration, mg/dL | All Subjects (n=774) | | | Men (n=510) | | | Women (n=264) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | HR (95% CI) | *P* Value | No. | HR (95% CI) | *P* Value | No. | HR (95% CI) | *P* Value |
| 1 | ≤3.90 | 162 | 1 [Reference] | | 39 | 1 [Reference] | | 123 | 1 [Reference] | |
| 2 | 3.91-4.60 | 140 | 0.88 (0.62-1.25) | .47 | 83 | 0.88 (0.52-1.49) | .63 | 57 | 0.90 (0.53-1.51) | .68 |
| 3 | 4.61-5.20 | 149 | 1.04 (0.73-1.47) | .83 | 113 | 1.17 (0.71-1.91) | .54 | 36 | 0.86 (0.45-1.62) | .63 |
| 4 | 5.21-6.20 | 165 | 0.80 (0.55-1.15) | .23 | 137 | 0.78 (0.48-1.29) | .33 | 28 | 1.34 (0.68-2.64) | .40 |
| 5 | ≥6.21 | 158 | 0.64 (0.44-0.94) | .02 | 138 | 0.67 (0.41-1.11) | .12 | 20 | 0.58 (0.23-1.50) | .26 |
| 1-SD increase in serum urate concentration | | | 0.82 (0.73-0.93) | .002 | | 0.81 (0.70-0.94) | .005 | | 0.89 (0.71-1.12) | .32 |

Abbreviations: CI, confidence interval; HR, hazard ratio.
[a]A 1-SD increase indicates an increase of 1.4 mg/dL. The HRs are adjusted for age, sex, and treatment group (deprenyl or placebo).

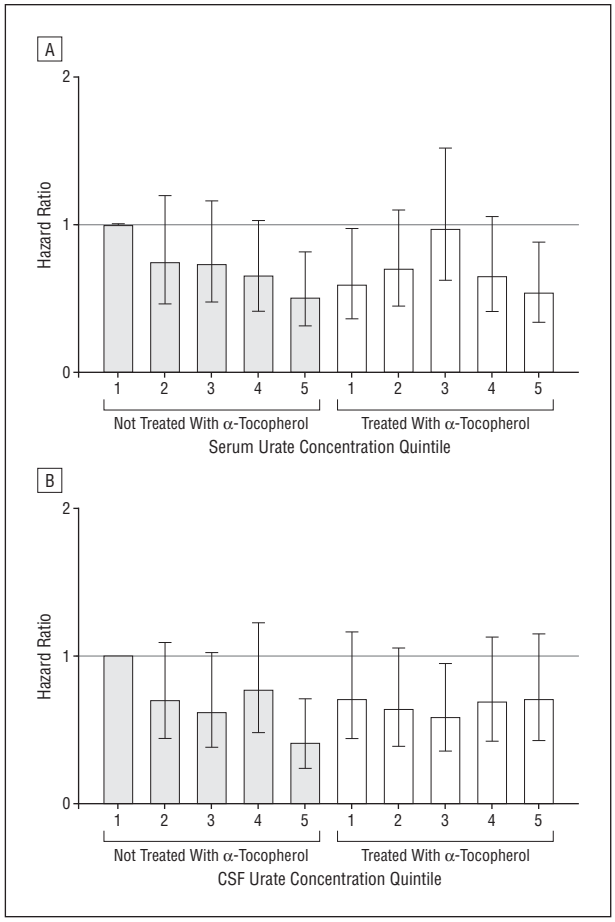


**Figure 1.** Hazard ratio for reaching the primary end point according to assignment to α-tocopherol (vitamin E) and quintile of baseline serum (A) or cerebrospinal fluid (CSF) (B) urate concentration (referenced to placebo-treated subjects in the lowest quintile). Error bars indicate 95% confidence intervals for hazard ratios adjusted for age, sex, and treatment group (deprenyl or placebo).

0.88 (95% CI, 0.71-1.08; *P*=.21) among subjects receiving α-tocopherol. In women, the corresponding HRs were 0.73 (95% CI, 0.52-1.02; *P*=.06) for subjects not receiving α-tocopherol and 1.04 (95% CI, 0.69-1.59; *P*=.84) for subjects receiving α-tocopherol. The interaction between α-tocopherol and serum urate concentration was nonsignificant for men and for women (*P* for interaction=.55 in men, and *P* for interaction=.06 in women). No significant interaction was found between serum urate concentration and deprenyl treatment; a decreasing HR with increasing serum urate concentration was found in the placebo-placebo and deprenyl-placebo groups but not in the placebo–α-tocopherol and deprenyl–α-tocopherol groups (eTable 1).

The change in UPDRS score between baseline and either the time of reaching the primary end point or the end of follow-up was available for 760 of the 774 subjects with baseline serum urate concentrations. Overall, the rate of UPDRS score change declined with increasing serum urate concentration (*P* for trend=.03). As observed previously for the primary end point, results were more robust in men, although there was no statistically significant interaction with sex. Among men, the adjusted rate of UPDRS score change declined from 14.8 points per year for subjects in the lowest quintile of serum urate concentration to 8.9 points per year for those in the highest quintile (*P* for trend=.03); comparable results among women were 11.0 and 8.2 points per year, respectively (*P* for trend=.35). The relationship between serum urate concentration and the rate of UPDRS score change was modified by α-tocopherol treatment (*P* for interaction=.009) (**Figure 2**A). In separate models, among subjects not assigned to receive α-tocopherol, the rate of UPDRS score change was 9.8 points per year lower in the highest serum urate concentration quintile than in the lowest quintile (*P*=.003), whereas no difference was observed for subjects assigned to receive α-tocopherol (0.5 points per year higher in the highest quintile as compared with the lowest quintile; *P*=.89). In analyses based on repeated measures, the overall association between higher urate levels at baseline and a slower rate of UPDRS score increase was even stronger (*P*=.001). There was also a significant interaction between urate concentration and α-tocopherol treatment (*P*=.003), and consistent with results observed in the primary analyses, higher levels of serum urate were strongly associated with a slower rate of UPDRS score increase among patients not treated with α-tocopherol (*P*=.001) but not in those treated with α-tocopherol (*P*=.37). No significant interaction was found between serum urate concentration and deprenyl treatment (eTable 2).

Two hundred eleven men (41.4%) and 81 women (30.7%) were identified as having died after 13 years of

follow-up. In men and women combined, after adjustment for deprenyl treatment, age, sex, and pack-years of smoking (**Table 3**) and after adjustment for deprenyl treatment, age, sex, pack-years of smoking, and cardiac comorbidity at baseline (**Table 4**), serum urate concentration was not significantly associated with mortality. In men, however, the relationship between serum urate concentration and mortality was a U-shaped curve, with the lowest mortality in the fourth quintile of urate concentration. In women, a suggestion of increased mortality at any urate concentration higher than that in the lowest quintile was not substantiated statistically. No significant interactions between serum urate concentration and α-tocopherol were found in analyses on mortality.

## CSF URATE CONCENTRATION

Mean urate concentrations in CSF collected at baseline were higher in men (0.42 mg/dL) than in women (0.28 mg/dL) and, as expected, were substantially lower than in serum.[21] Despite the lower concentrations of CSF urate, a strong correlation was found between CSF and serum urate concentrations ($r=0.73$; $P<.001$).

The primary clinical end point of disability was reached by 342 of the 713 subjects (48%) for whom CSF urate concentrations were available. Overall, the HR of reaching the primary end point of disability was significantly lower among individuals with higher concentrations of CSF urate. The HR comparing subjects in the highest quintile of CSF urate concentration with those in the lowest quintile was 0.65 (95% CI, 0.44-0.96; $P=.03$); the HR associated with a 1-SD increase in CSF urate concentration was 0.89 (95% CI, 0.79-1.02; $P=.09$) (**Table 5**). Results were not significantly different by sex, although a strong interaction was found between α-tocopherol assignment and CSF urate concentration ($P$ for interaction $=.009$) (Figure 1B). As for serum urate concentration, a significant decrease in the HRs for the primary end point with increasing CSF urate concentration was observed only among subjects not receiving α-tocopherol. The HR corresponding to a 1-SD increase in CSF urate concentration was 0.77 (95% CI, 0.62-0.96; $P=.02$) among men not treated with α-tocopherol and 1.10 (95% CI, 0.90-1.34; $P=.34$) among those receiving α-tocopherol. In women, the corresponding HRs were 0.64 (95% CI, 0.40-1.03; $P=.07$) for subjects not assigned to α-tocopherol and 0.77 (95% CI, 0.43-1.37; $P=.37$) for subjects treated with α-tocopherol. No significant interaction was found between CSF urate concentration and deprenyl treatment (eTable 3).

The change in UPDRS score between baseline and either the time of reaching the primary end point or the end of follow-up was available for 702 of the 713 subjects with baseline CSF urate concentrations. Overall, the rate of UPDRS score change was not related significantly to CSF urate concentration. As observed for serum urate concentration, however, the relationship between CSF urate concentration and the rate of UPDRS score change was modified by α-tocopherol treatment ($P$ for interaction $=.04$) (Figure 2B). Among subjects not treated with α-tocopherol, the rate of UPDRS score change declined with increasing CSF urate concentrations ($P$ for trend $=.05$). Conversely, randomization to α-tocopherol treatment appeared to lower the rate of UPDRS score change among subjects in the lowest quintile of urate concentration measured either in CSF (Figure 2B) or in serum (Figure 2A) but not among those with higher urate concentrations. No significant interaction was found between CSF urate concentration and deprenyl treatment (eTable 4).

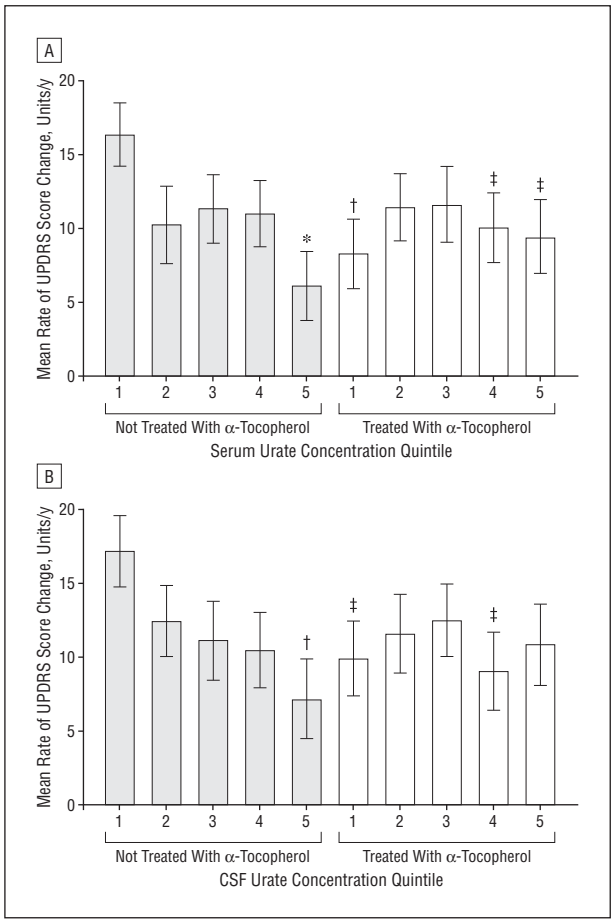


**Figure 2.** Mean annualized rate of Unified Parkinson's Disease Rating Scale (UPDRS) score change according to assignment to α-tocopherol (vitamin E) and quintile of baseline serum (A) or cerebrospinal fluid (CSF) (B) urate concentration. Error bars indicate standard errors of the mean adjusted for age, sex, and treatment group (deprenyl or placebo). *Significantly different from the placebo-treated subjects in the lowest quintile ($P<.001$). †Significantly different from the placebo-treated subjects in the lowest quintile ($P<.01$). ‡Significantly different from the placebo-treated subjects in the lowest quintile ($P<.05$).

## COMMENT

Among subjects with early PD participating in a large randomized trial, we found that both serum and CSF urate concentrations measured at baseline were inversely related to clinical progression of PD. The internal consistency of the results across the primary and secondary end points supports their validity. These findings, like data from a similar early PD trial (the Parkinson Research Examination of CEP-1347 Trial [PRECEPT] study),[13] demonstrate a robust link between blood urate concentrations and the rate of clinical progression in PD. In addition, the association of CSF urate concentration with disease progression

**Table 3. Hazard Ratios for Death From Any Cause According to Common Quintiles of Baseline Serum Urate Concentration, Adjusted for Age, Sex, Treatment Group (Deprenyl or Placebo), and Pack-Years of Smoking**

| Serum Urate Concentration Quintile | All Subjects (n=768) | | Men (n=504) | | Women (n=264) | |
|---|---|---|---|---|---|---|
| | HR (95% CI) | *P* Value | HR (95% CI) | *P* Value | HR (95% CI) | *P* Value |
| 1 | 1 [Reference] | | 1 [Reference] | | 1 [Reference] | |
| 2 | 1.17 (0.77-1.78) | .47 | 0.66 (0.38-1.15) | .14 | 1.68 (0.90-3.11) | .10 |
| 3 | 1.20 (0.78-1.83) | .41 | 0.66 (0.38-1.12) | .12 | 1.30 (0.64-2.66) | .47 |
| 4 | 1.11 (0.72-1.72) | .62 | 0.60 (0.35-1.02) | .06 | 1.88 (0.89-3.97) | .10 |
| 5 | 1.48 (0.96-2.27) | .06 | 0.89 (0.53-1.50) | .67 | 1.96 (0.89-4.33) | .10 |

Abbreviations: CI, confidence interval; HR, hazard ratio.

**Table 4. Hazard Ratios for Death From Any Cause According to Common Quintiles of Baseline Serum Urate Concentration, Adjusted for Age, Sex, Treatment Group (Deprenyl or Placebo), Pack-Years of Smoking, and Cardiac Comorbidity at Baseline**

| Serum Urate Concentration Quintile | All Subjects (n=768) | | Men (n=504) | | Women (n=264) | |
|---|---|---|---|---|---|---|
| | HR (95% CI) | *P* Value | HR (95% CI) | *P* Value | HR (95% CI) | *P* Value |
| 1 | 1 [Reference] | | 1 [Reference] | | 1 [Reference] | |
| 2 | 1.12 (0.73-1.71) | .61 | 0.63 (0.36-1.10) | .10 | 1.66 (0.90-3.08) | .11 |
| 3 | 1.14 (0.74-1.74) | .56 | 0.63 (0.37-1.08) | .09 | 1.29 (0.63-2.64) | .49 |
| 4 | 1.05 (0.68-1.62) | .83 | 0.56 (0.33-0.95) | .03 | 1.85 (0.87-3.93) | .11 |
| 5 | 1.38 (0.89-2.12) | .15 | 0.83 (0.49-1.39) | .47 | 1.89 (0.83-4.30) | .13 |

Abbreviations: CI, confidence interval; HR, hazard ratio.

**Table 5. Hazard Ratios for Reaching the Primary End Point According to Common Quintiles of Baseline Cerebrospinal Fluid Urate Concentration or Corresponding to a 1-SD Increase in Cerebrospinal Fluid Urate Concentration[a]**

| CSF Urate Concentration Quintile | CSF Urate Concentration, mg/dL | All Subjects (n=713) | | | Men (n=473) | | | Women (n=240) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | HR (95% CI) | *P* Value | No. | HR (95% CI) | *P* Value | No. | HR (95% CI) | *P* Value |
| 1 | ≤0.23 | 143 | 1 [Reference] | | 38 | 1 [Reference] | | 105 | 1 [Reference] | |
| 2 | 0.24-0.32 | 143 | 0.78 (0.55-1.10) | .16 | 81 | 1.05 (0.61-1.81) | .85 | 62 | 0.65 (0.38-1.11) | .11 |
| 3 | 0.32-0.39 | 144 | 0.70 (0.48-1.01) | .06 | 109 | 0.96 (0.57-1.63) | .89 | 35 | 0.51 (0.26-1.03) | .06 |
| 4 | 0.39-0.50 | 142 | 0.84 (0.58-1.22) | .36 | 117 | 1.08 (0.64-1.80) | .78 | 25 | 0.72 (0.34-1.55) | .40 |
| 5 | ≥0.51 | 141 | 0.65 (0.44-0.96) | .03 | 128 | 0.85 (0.51-1.43) | .54 | 13 | 0.47 (0.16-1.39) | .17 |
| 1-SD increase in CSF urate concentration | | | 0.89 (0.79-1.02) | .09 | | 0.93 (0.80-1.07) | .28 | | 0.79 (0.57-1.10) | .17 |

Abbreviations: CI, confidence interval; CSF, cerebrospinal fluid; HR, hazard ratio.
[a]A 1-SD increase indicates an increase of 0.16 mg/dL. The HRs are adjusted for age, sex, and treatment group (deprenyl or placebo).

strengthens the possibility that brain urate concentration (or its determinants) might protect against the neurodegeneration of PD. Taken together, these data establish urate as the first molecular predictor of clinical progression in PD and provide a rationale for investigating the possibility that a therapeutic increase of urate in patients with PD might act favorably to slow the disease course. Interestingly, the inverse relationship between urate concentration and clinical progression was not observed among patients randomized to α-tocopherol at a dosage of 2000 IU/d, suggesting that there may be an interaction between these antioxidants.

There is strong evidence that oxidative stress and nitrative stress are major pathogenetic mechanisms in PD.[13,22,23] Urate is an effective antioxidant,[1] peroxynitrite scavenger,[24-27] iron chelator,[28] and ascorbate stabilizer.[29] In cellular models of PD neurodegeneration, urate can reduce oxidative stress, mitochondrial dysfunction, and cell death occurring spontaneously in culture or induced by the pesticide rotenone, 1-methyl-4-phenylpyridinium, glutamate, and iron ions.[30-32] Although urate appears to have the potential for neuroprotection, it is possible that the predictive association between urate concentration and PD progression reflects instead the effect of a urate precursor, such as adenosine or inosine, or another determinant of systemic and CSF urate concentrations.

As compared with serum urate concentration, the weaker association of CSF urate concentration to clinical progression of PD may seem at odds with the hypothesis that urate (or its metabolic precursors) exerts a beneficial effect through presence in the central nervous system. The CSF urate concentrations, how-

ever, display a strong caudorostral gradient from the lumbar space, with lumbar region values approximately 50% higher than those arising at the cisterna magna (brainstem) level.[33,34] Although we consistently used CSF aliquots obtained from the 18th to 20th milliliter of CSF flow, variations in CSF circulation patterns between patients[35]—along with freezer storage for 20 years—may have contributed to a reduction of the accuracy of this measure compared with assays of freshly collected serum samples. In addition to technical variability, substantial biological differences between the urate in CSF sampled from the subarachnoid space and that in the degenerating neurons themselves may lessen the strength of a CSF urate concentration–clinical progression correlation in PD.

The finding that the inverse relationship between urate concentration and clinical progression of PD was modified by α-tocopherol treatment was unforeseen because, as originally reported, no favorable effect of α-tocopherol on PD progression was found among study participants in the DATATOP trial.[16] The mechanisms for a possible interaction between urate and α-tocopherol remain uncertain. Although hydrophilic (eg, urate) and hydrophobic (eg, α-tocopherol) antioxidants target different subcellular compartments, their functional interactions have been described.[36,37] Further, α-tocopherol at doses commonly used in vitamin supplements may reduce concentrations of other endogenous antioxidants,[38,39] and α-tocopherol at high doses may have pro-oxidant rather than antioxidant effects.[40,41] Alternatively, a simple competitive interaction or "ceiling effect" may have contributed to the observed lack of α-tocopherol benefits among patients with PD with higher urate concentrations as well as to the loss of the inverse association between urate concentration and PD progression among those receiving supplemental α-tocopherol. Regardless of the mechanism for a possible interaction between α-tocopherol and urate, our results raise the possibility that such an interaction may have obscured a protective effect of α-tocopherol among those subjects with low baseline concentrations of urate in the DATATOP trial. Further investigations are therefore needed to consider the possibility that α-tocopherol supplementation may be beneficial in individuals with low urate concentrations.

Serum urate may also affect the progression of cognitive impairment in that higher concentrations seem to be associated with slower rates of cognitive decline and lower risk of dementia.[42-44] As in the present study, among participants in a randomized trial, this association was observed in patients treated with placebo but not in those treated with α-tocopherol.[43] Higher serum urate concentration has also been linked to a lower rate of worsening in Huntington disease.[45] Although each of these neurodegenerative disorders manifests differently from PD, the relationships between urate concentration and these disorders may be indicative of a more general influence of urate (or its precursors) on neuronal cell death.

The main results of this study are strikingly consistent with those recently reported from the PRECEPT study.[13] Although the overall inverse relationship between serum urate concentration and the clinical progression of PD was greater in the PRECEPT study than in the DATATOP study, results among subjects in the DATATOP study not assigned to α-tocopherol were virtually identical to those observed in the PRECEPT study (which did not include an α-tocopherol treatment arm). In both trials, HRs for risk of disability progression showed a decline in patients whose values were higher than the median concentration but still within the normal range of serum urate concentrations. Moreover, in both trials, the concentration-dependent inverse relationship was robust in men but weak and nonsignificant among women. This consistent difference between men and women could result in part from a biological effect of sex on urate mechanisms in PD[46] or could simply reflect the small number of women with urate concentrations high enough to slow disease progression if urate were protective.

A potentially therapeutic effect of elevating serum urate concentration warrants consideration. Urate levels can be elevated by dietary means, including increased intake of fructose[47-49] or purines,[50] or by pharmacological means. The latter may include administration of the purine metabolite and urate precursor inosine, which is being investigated as a therapy for multiple sclerosis.[26,27] The potential benefit of elevating urate concentration in individuals with PD, however, has to be weighed against possible adverse effects, which may include an increased risk of hypertension, coronary heart disease, and stroke[6,51-53] in addition to the known risks of gout and urolithiasis. Available data are therefore insufficient to support a therapeutic recommendation.

The discovery of a urate link to PD progression was achieved through additional analyses of 2 rigorously conducted clinical trials whose databases were made available to test an unforeseen hypothesis months[54] or decades[18,19] after conclusion of the primary investigations. These latent insights highlight a broader opportunity to achieve further advances through explorations of the growing repository of high-quality data collected from neuroprotection trials of PD and other neurodegenerative disorders.

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Appendix R

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# Inactivation of neuronal forebrain $A_{2A}$ receptors protects dopaminergic neurons in a mouse model of Parkinson's disease

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## Abstract

Adenosine $A_{2A}$ receptors antagonists produce neuroprotective effects in animal models of Parkinson's disease (PD). Since neuroinflammation is involved in PD pathogenesis, both neuronal and glial $A_{2A}$ receptors might participate to neuroprotection. We employed complementary pharmacologic and genetic approaches to $A_{2A}$ receptor inactivation, in a multiple 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, to investigate the cellular basis of neuroprotection by $A_{2A}$ antagonism. MPTP·HCl (20 mg/kg daily for 4 days) was administered in mice treated with the $A_{2A}$ antagonist SCH58261, or in conditional knockout mice lacking $A_{2A}$ receptors on forebrain neurons (fbn$A_{2A}$KO mice). MPTP induced partial loss of dopamine neurons in substantia nigra pars-compacta (SNc) and striatum (Str), associated with increased astroglial and microglial immunoreactivity in these areas. Astroglia was similarly activated one, three and seven days after MPTP administration, whereas maximal microglial reactivity was detected on day one, returning to baseline seven days after MPTP administration. SCH58261 attenuated dopamine cell loss and gliosis in SNc and Str. Selective depletion of $A_{2A}$ receptors in fbn$A_{2A}$KO mice completely prevented MPTP-induced dopamine neuron degeneration and gliosis in SNc, and partially counteracted gliosis in Str. Results provide evidence of a primary role played by neuronal $A_{2A}$ receptors in neuroprotective effects of $A_{2A}$ antagonists in a multiple MPTP injections model of PD. With the symptomatic antiparkinsonian potential of several $A_{2A}$ receptor antagonists being pursued in clinical trials, the present study adds to the rationale for broader clinical benefit and use of these drugs early in the treatment of PD.

### Keywords

adenosine; MPTP; microglia; astroglia; tyrosine hydroxylase

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Since the initial demonstration that caffeine can attenuate MPTP-induced toxicity, a neuroprotective role of $A_{2A}$ receptor blockade has been suggested in several experimental models of Parkinson's disease (PD) (Chen et al., 2001; Schwarzschild et al., 2006). In rodents different $A_{2A}$ receptor antagonists have been shown to counteract the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), as well as dopamine depletion in the striatum (Str), induced by acute administration of systemic MPTP or acute infusion of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (Chen et al., 2001 Chen et al., 2002; Ikeda et al., 2002; Pierri et al., 2005). Moreover, loss of striatal dopamine induced by acute MPTP was attenuated by the global genetic deletion of $A_{2A}$ receptors in $A_{2A}$ knockout (KO) mice (Chen et al., 2001). In contrast to acute models, neuroprotection by $A_{2A}$ antagonists in the neurotoxicity induced by multiple MPTP administration was never evaluated. On the other hand, previous studies have shown that acute or multiple injections MPTP delivery result in different histopathological features and different modes of cell death (Schmidt and Fergen, 2001). Interstingly, $A_{2A}$ receptor antagonism or gene KO has been found to be neuroprotective in different models of neurodegeneration, such as Alzheimer's disease, Huntington's disease and cerebral ischemia (Chen et al., 1999; Dall'Igna et al., 2003; Fink et al., 2004; Melani et al., 2003).

$A_{2A}$ receptors are enriched in the Str, where they are located either postsynaptically in striatopallidal neurons, or presynaptically in nerve terminals (Rebola et al., 2005; Rosin et al., 1998; Schiffman et al., 1991; Svenningsson et al., 1999). Moreover, $A_{2A}$ receptors are expressed at low level in other forebrain structures, such as cortex and hippocampus, whereas little or no $A_{2A}$ receptor immunoreactivity has been detected in dopaminergic neurons in the SNc (Rosin et al., 1998). Besides neurons, non-neuronal cell types such as microglia and astroglia also express $A_{2A}$ receptors (Fiebich et al., 1996; Saura et al., 2005).

The mechanism through which $A_{2A}$ receptor antagonists achieve neuroprotection in PD models has not been elucidated. Given the cellular distribution of $A_{2A}$ receptors in different cell types in brain, neuroprotection by $A_{2A}$ receptor blockade may be achieved through an action on receptors located either on neurons or on glial cells. Attenuation of gliosis by $A_{2A}$ receptor antagonists in the Str and SNc of mice acutely treated with MPTP, suggests that reduction of neuroinflammation may be involved (Ikeda et al., 2002; Pierri et al., 2005). Moreover, it was recently suggested that $A_{2A}$ receptors located on glial cells may play a role in neuroprotection mediated by $A_{2A}$ antagonists against an acute MPTP-induced striatal dopamine depletion (Yu et al, 2008).

In order to get insight into the mechanism by which $A_{2A}$ receptor antagonists induce neuroprotection, the present study evaluated 1) the neuroprotective activity of an $A_{2A}$ antagonist in a mouse model of PD obtained by a multiple injections MPTP delivery; 2) the role of neuronal $A_{2A}$ receptors on this effect and 3) whether neuronal $A_{2A}$ receptor blockade might affect glial response to MPTP. To this aim, we treated mice with MPTP plus the $A_{2A}$ antagonist SCH58261, or administered MPTP to genetically manipulated mice selectively lacking $A_{2A}$ receptors in forebrain neurons (fbn$A_{2A}$KO mice) (Bastia et al., 2005; Shen et al., 2008). Neuronal damage was evaluated in the SNc and Str using tyrosine hydroxylase (TH) immunohistochemistry, whereas the inflammatory response in the SNc and Str was studied through analysis of glial fibrillary acidic protein (GFAP) and CD11b immunoreactivity as markers of astroglial and microglial cells, respectively.

The present study provides evidence of a primary role played by forebrain $A_{2A}$ receptors in the neuroprotection that $A_{2A}$ receptor antagonists confer against dopaminergic neuron degeneration and glial activation induced by repeated MPTP.

# Methods

## Animals and treatments

Male C57BL/6J mice (Charles River, Italy) were used for experiments involving pharmacological treatments with the $A_{2A}$ receptor antagonist. Mice were housed 5 per cage, with a 12:12 hrs light/dark cycle and with food and water *ad libitum*. Experiments were conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health.

Pharmacological treatment: Male C57BL/6J mice (25–30 gm, 3 months old), received a multiple injections treatment with vehicle (N= 15), MPTP·HCl (20 mg/kg i.p.) once a day for 4 days (N = 18), or the $A_{2A}$ antagonist SCH58261 (0.5 mg/kg i.p.) twice a day plus MPTP (20 mg/kg i.p.) once a day for 4 days (N = 23). SCH58261 was injected half an hour before MPTP administration. After MPTP treatment discontinuation SCH58261 treatment continued once a day, until sacrifice, which occurred 1, 3 or 7 days after MPTP treatment. Injections were made at 8 a.m. and 8 p.m.

Postnatal forebrain neuron conditional $A_{2A}$KO mice were generated using the Cre/*loxP* system based on the specificity of *CaMKIIα* promoter (Bastia et al, 2005, Yu et al, 2008; see also Additional Informations (A.I.) for online version). Fbn$A_{2A}$KO mice and littermate controls were treated with repeated MPTP (n=18 or n=15) or vehicle (n=12 or n=10) as described above. Experiments with the $A_{2A}$ antagonist (see below) showed that loss of TH-immunoreactive cells was stable 1, 3 and 7 days after MPTP treatment, and that CD11b immunoreactivity was greatest 1 and 3 days post-MPTP treatment. Based on these considerations, neuroprotection and glial reactivity parameters in transgenic mice were evaluated 3 days after MPTP treatment.

## Drugs

MPTP·HCl (Sigma-Aldrich, Italy or USA) was dissolved in 0.9% saline in a volume of 0.1 ml/10 g; SCH58261 (kindly provided Prof Baraldi, Ferrara) was suspended in 0.5% of methylcellulose.

## Immunohistochemistry

Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) or Avertin (0.1 ml/10 g i.p.) prior to transcardial perfusion with 20 ml of saline and 60 ml of 4% paraformaldehyde. Brains were removed and post-fixed for 2 hour. Adjacent coronal sections (50 μm) from the Str and SNc were cut on a vibratome and stored at −20°C in an antifreeze medium until use (Schintu et al, 2009). For TH, GFAP and CD11b immunostaining, adjacent sections were processed as described (Schintu et al, 2009; See also A.I.).

## Analysis and statistics

Images were digitized (videocamera Pixelink PL-A686) under constant light conditions to standardize the measurements. Immunostained sections containing left and right SNc, were captured at 10X magnification (the entire SNc, corresponding to three frames, was digitized for the analysis). Immunostained sections of bilateral striata were captured at 20X magnification. One portion from the dorsolateral Str and one from the ventromedial Str (520 μm × 380 μm) were analysed. For each animal, three sections corresponding to rostral (within −2.90/−3.20 mm from bregma), medial (−3.20/−3.50) and caudal (−3.50/−3.80) SNc levels, and three sections corresponding to rostral (within 1.20/0.90 mm from bregma), medial (0.90/0.60) and caudal (0.60/0.30) Str (accordingly to Mouse Brain Atlas, Paxinos and Franklin, 2001) were analysed for each protein marker evaluated in the study. *TH and*

*GFAP analyses*: since the number of cells was different in the three SNc and Str levels analysed, for each mouse the number of TH- or GFAP-positive cells/level was first normalized with respect to the vehicle. Individual values from the 3 levels were then averaged to generate a mean. Adjacent SNc sections were Nissl-stained, in order to confirm cell loss in this area.

The SNc from three mice randomly selected from each experimental group were counterstained with cresyl violet for evaluation by unbiased stereological counting, and the mean of TH-ir and Nissl-stained neurons per $mm^3$ ± SEM was calculated (see A.I).

*CD11b analysis*: images were digitized in a grey scale, and CD11b immunostaining was evaluated with the analysis program Scion Image. A threshold, the value of which was set above the mean value ± S.E.M. of the background, was applied for background-correction. Inside each frame, the area occupied by grey values above the threshold was automatically calculated. For each level of SNc or Str, the obtained value was first normalized with respect to vehicle; the individual values from the 3 levels were then averaged to generate a mean.

Results from mice treated with MPTP plus SCH58261, or from fbn$A_{2A}$KO mice were statistically compared with a two-factors ANOVA, followed by Tukey's *post hoc* test, for comparison between experimental groups.

## Results

### $A_{2A}$ receptor antagonist prevents dopaminergic cell loss in the SNc

In C57BL/6J mice, MPTP treatment induced a partial dopaminergic neurons degeneration in the SNc (Fig 1A, B and Tab 1). Counting of Nissl-stained cells confirmed this result (Tab 1). Dopaminergic neurons loss was statistically significant 1 day after MPTP treatment (N= 5) and remained significant after 3 (N=5) and 7 days timepoints (N=8). Combined treatment with $A_{2A}$ antagonist SCH58261 plus MPTP, attenuated TH-positive neurons loss in the SNc at 1 (N= 5), 3 (N=5) and 7 days (N=13) (Fig 1A, B and Tab 1), as confirmed by Nissl-staining (Tab 1). Two-factors ANOVA showed a significant effect of treatment (see Suppl. Table 1 in A.I. for corresponding F and P values). In the Str, MPTP-induced decrease in TH-immunoreactivity was significantly attenuated by treatment with the $A_{2A}$R antagonist, as measured three days after treatment (Fig 2).

### $A_{2A}$ receptor antagonist inhibits astroglia and microglia activation

In brain sections from vehicle-treated mice, few GFAP-positive cells (Fig 3), and a low CD11b immunostaining (Figs 3, 4), were detected in the SNc and Str. Repeated MPTP treatment induced an increase in GFAP and CD11b-positive cells in both the SNc and Str of C57BL/6J mice.

GFAP-positive cells displayed a highly branched morphology with tiny processes and a small body in control sections, and became hypertrophic in response to MPTP treatment (Fig 3A); moreover, CD11b-positive cells were ramified at baseline, but took on an ameboid aspect after MPTP treatment (Fig 4A), indicative of astroglial and microglial activation. In both the SNc and Str, GFAP immunolabelling was of similar intensity 1, 3 and 7 days post-MPTP treatment (Fig 3B). Combined SCH58261 plus MPTP treatment, completely prevented the increase in GFAP immunoreactivity in the SNc and partially prevented it in the Str, at 1, 3 and 7 days after MPTP treatment (Fig 3B). Two-factors ANOVA for GFAP analysis showed a significant effect of the treatment at all time points analyzed in the SNc and in the Str (see Suppl. Table 1 in A.I.).

CD11b immunolabelling was highest 1 day after MPTP, gradually declining to basal levels after 7 days in both SNc and Str (Fig 4A, B). Combined SCH58261 plus MPTP treatment completely prevented the increase in CD11b immunoreactivity in the SNc 1 and 3 days after MPTP treatment (Fig 4A,B). In the Str, SCH58261 partially prevented MPTP-induced increase in CD11b at 1 day, and totally prevented it at 3 days after MPTP treatment. Two-factors ANOVA and *post hoc* analysis for CD11b revealed a significant effect of treatment, time, and a treatment/time interaction, 1 and 3 days after MPTP administration in the SNc and in the Str (se Suppl. Table 1 in A.I.).

### Dopaminergic cell loss is prevented in fbn$A_{2A}$KO mice

In fbn$A_{2A}$WT mice, repeated MPTP treatment induced a significant loss of dopamine neurons in the SNc (Fig 5A, B and Tab 1). This result was confirmed by a reduction in Nissl-stained cells (Tab 1 and see right insert in fig 5A). In contrast, in fbn$A_{2A}$KO mice, repeated MPTP treatment did not result in a decrease in TH-immunolabelling in the SNc (Fig 5A, B and Tab 1). Two-factors ANOVA revealed a significant effect of treatment and genotype (see Suppl. Table 1 in A.I. for corresponding F and P values). In the Str of fbn$A_{2A}$KO mice repeated MPTP reduced TH immunolabelling to a lesser extent as compared with fbn$A_{2A}$WT controls (Fig 2).

### Astroglial and microglial activation is attenuated in fbn$A_{2A}$KO mice

GFAP immunoreactivity in the SNc and Str was significantly enhanced in MPTP-treated fbn$A_{2A}$WT mice (Fig 6A, B). In contrast, in fbn$A_{2A}$KO mice, MPTP-induced increase in GFAP immunoreactivity was totally prevented in the SNc, and partially prevented in Str (Fig 6A, B). In both brain regions two-factors ANOVA revealed a significant effect of treatment, genotype and a significant treatment/genotype interaction (see Suppl. Table 1 in A.I.).

CD11b immunolabelling in the SNc and Str was significantly enhanced in MPTP-treated fbn$A_{2A}$WT mice (Fig 7A, B). In fbn$A_{2A}$KO mice CD11b activation was totally prevented in the SNc and Str (Fig 7A, B). Two-factors ANOVA revealed a significant effect of treatment, genotype and a treatment/genotype interaction (see Suppl. Table 1 in A.I.).

## Discussion

Antagonism of adenosine $A_{2A}$ receptors or their selective deletion in forebrain neurons produced similar protection of TH-positive nigral neurons in a multiple MPTP injections mouse model of PD. These complementary pharmacological and genetic means of $A_{2A}$ receptor disruption also attenuated the neurotoxin-triggered activations of astroglia and microglia along the nigrostriatal pathway. Together these data provide evidence that therapeutically accessible $A_{2A}$ receptors located on forebrain neurons play a critical role in nigral dopaminergic neuron degeneration and inflammatory processes in the multiple MPTP injections mouse model of PD.

### Pharmacological blockade of $A_{2A}$ receptors prevents MPTP-induced dopaminergic neuron degeneration and glial activation

Systemic administration of the $A_{2A}$ receptor antagonist SCH58261 prevented the degeneration of nigrostriatal TH-positive neurons induced by repeated MPTP exposure in mice. Changes in number of TH-positive neurons correlated with changes in Nissl-stained (cresyl violet-positive) cells, indicating that MPTP treatment resulted in actual loss of dopaminergic neurons, which were rescued by SCH58261.

A neuroprotective effect of $A_{2A}$ receptor antagonists was previously observed upon acute administration of high MPTP doses in mice (Chen et al., 2001; Ikeda et al., 2002; Pierri et al., 2005; Yu et al, 2008). Here we report that neuroprotection with $A_{2A}$ antagonism can also be achieved upon multiple low-doses MPTP exposure. A number of studies have provided evidence that a repeated daily MPTP administration protocol similar to the one used here, presents histopathological features that more closely reproduce the human PD neuropathology, including apoptotic death of dopaminergic neurons (Jackson-Lewis et al., 1995; Tatton & Kish, 1997). Therefore, the present study further substantiates the neuroprotective potential of $A_{2A}$ antagonism in PD. To this regard, it is noteworthy that $A_{2A}$R antagonists were shown to inhibit apoptotic neuronal death in hippocampal neurons (Silva et al, 2007).

Based on their differential location in the Str or other brain regions, $A_{2A}$ receptors may hold different levels of expression and intracellular signalling, reflecting $A_{2A}$ receptor multiple functions (Kull et al, 2000; Pedata et al, 2003; Rebola et al, 2005; Rosin et al, 2003; Shen et al, 2008). According to such varied roles of the $A_{2A}$ receptor, diverse effects have been attributed to $A_{2A}$ antagonists, ranging from symptomatic antiparkinsonian actions to neuroprotection in various neurodegenerative conditions (Alfinito et al., 2003; Blum et al., 2003; Chen et al., 1999; Melani et al., 2003; Monopoli et al., 1998; Popoli et al., 2002). Motor effects of $A_{2A}$ receptor antagonists are likely mediated by $A_{2A}$ receptors located on striatal neurons projecting to globus pallidus, whereas several mechanisms have been hypothesized for their neuroprotective effects, involving either neuronal or glial $A_{2A}$ receptors, though no single mechanism has yet been proven to prevail (Carta et al., 2003; Chen et al., 2001; Huang et al, 2006; Melani et al., 2003; Popoli et al., 1995; Pedata et al., 2003; Schwarzschild et al, 2006; Yu et al, 2008). Noticeably, in the present study neuroprotection by SCH58261 was achieved at doses similar to those effective in other neurodegenerative conditions, but several times lower than doses displaying a symptomatic efficacy in PD (Chen et al., 2001; Dall'Igna et al., 2003; Melani et al., 2003; Pinna et al., 2007), supporting the concept that different mechanisms might account for $A_{2A}$-mediated neuroprotection or symptomatic effects.

SCH58261 fully prevented astroglia and microglia activation in the SNc, while only partially inhibiting astroglia and microglia reactivity in the striatum, in line with a partial protection of dopaminergic terminals. Noteworthy, $A_{2A}$ receptor antagonism prevented both astroglia and microglia activation in the SNc and Str at all time-points evaluated, in accordance with blockade of neurodegeneration.

Though the mechanism through which $A_{2A}$ receptor blockade produces neuroprotective effects in PD models is unclear, the modulation of neuroinflammation has been proposed as a likely target for neuroprotection (Hunot and Hirsch, 2003). Several findings have suggested that neuroinflammation may play an active role in the pathogenesis of neurodegeneration in PD, since focal inflammation has been described in in the SNc of PD patients and MPTP-treated primates (Barcia et al., 2004; McGeer et al., 1988). Intriguingly, blockade of microglia reactivity in mice rescued dopamine neurons from acute MPTP toxicity (Wu et al., 2002). Moreover, in mice acutely treated with MPTP, dopamine neuron neuroprotection by pre-treatment with an $A_{2A}$ antagonist was associated with an attenuation of astroglia and microglia activation in SNc and Str (Ikeda et al., 2002; Pierri et al., 2005), consistent with a causal relation between the two events.

## Selective deletion of neuron specific forebrain (fbn) $A_{2A}$ receptors prevents MPTP-induced dopamine neuron degeneration and glial activation

Previous studies evaluating $A_{2A}$ receptor-mediated neuroprotection have hypothesised several mechanisms that might underlie this process. In order to determine the role of

neuronal *versus* glial $A_{2A}$ receptors in neuroprotection of dopamine neurons, we exploited genetically modified mice with selective depletion of $A_{2A}$ receptors from forebrain neurons (Bastia et al., 2005; Shen et al, 2008). Importantly, in the fbn$A_{2A}$KO mice, deletion of $A_{2A}$ receptor is not only spatially restricted (to forebrain $A_{2A}$ receptor) but is also temporally limited to postnatal $A_{2A}$ receptors (Bastia et al., 2005; Yu et al., 2008), thus avoiding potential confounds of compensatory responses to $A_{2A}$ receptor gene disruption during development as might occur in constitutive $A_{2A}$ knockout mice.

Our results revealed that selective deletion of $A_{2A}$ receptors from forebrain neurons totally prevented dopaminergic neuron loss in the SNc following multiple MPTP injections, while partially preventing damage to striatal dopaminergic terminals. $A_{2A}$R deletion provided a greater protection of SNc neurons than the $A_{2A}$R antagonist, as expected from a permanent as compared to a temporal pharmacological blockade of the receptor, in line with the reported half-life of SCH58261 of 2–3 h.

$A_{2A}$ receptors are located both pre- and post-synaptically in striatal and cortical neurons, and are expressed in microglial as well as astroglial cells (Cunha, 2001; Kust et al., 1999; Nishizaki et al, 2004; Rebola et al., 2005; Rosin et al., 2003). Positive modulation of postsynaptic signalling as well as of pre-synaptic release of neurotransmitters as glutamate and acetylcholine by $A_{2A}$ receptors have been described (Fredholm et al., 2003; Fuxe et al., 2003; Marchi et al., 2002; Popoli et al., 1995; Schiffmann et al., 2007; Schwarzschild et al, 2006). In addition, $A_{2A}$ receptors interfere with glia-mediated synthesis and release of neurotoxic factors such as COX-2, prostaglandins, nitric oxide and glutamate, which have been hypothesised to play central roles in inflammatory processes and neuronal damage (Fiebich et al., 1996; Li et al., 2001; Saura et al., 2005).

The present study, by showing that selective deletion of $A_{2A}$ receptors from forebrain neurons protects dopaminergic neurons from MPTP toxicity, endorses a primary role of neuronal receptors in mediating neuroprotection in this multiple injections MPTP model of PD. Since very low levels of $A_{2A}$ receptors are expressed by dopaminergic neurons in the SNc, it is unlikely that a direct action at this level might mediate neuroprotection from MPTP toxicity in the SNc. Rather, an indirect effect at the pre-synaptic level, through an inhibition of $A_{2A}$-mediated glutamate release, which contribute to neuronal damage, could be envisaged (Aguirre et al., 2005; Battaglia et al., 2004; Monopoli et al., 1998; Popoli et al., 2002). Interestingly, recent studies have reported a tight cross-talk between adenosine and GDNF receptors, resulting in a fine modulation of glutamate and dopamine release (Gomes et al, 2006; 2009). In the Str, $A_{2A}$R blockade would impair GDNF-stimulated increase of corticostriatal glutamate release, thus providing a beneficial effect on neurodegeneration. In addition, $A_{2A}$ receptor antagonism on striatopallidal or subthalamic (STN) neurons might be protective from MPTP toxicity by modulating excessive activation of subthalamic nucleus, thereby reducing excitotoxic glutamate efflux to SNc neurons (Wallace et al., 2007).

A study by Yu and colleagues reported that fbn$A_{2A}$KO mice from the same line as used here, were not protected from striatal dopamine loss in response to acute MPTP exposure (in a single dose or multiple doses over 4 hr; Yu et al., 2008). These results open to several compelling interpretations. First, the cellular basis of $A_{2A}$ receptor-dependence of MPTP toxicity might vary depending on the duration of toxin exposure, in line with the different type of neurotoxicity produced by acute as compared to subchronic MPTP. Moreover, it should be taken into account the different parameters used to evaluate nigrostriatal neurons damage. The drop of striatal dopamine levels assessed by Yu et al. may reflect functional injury to dopaminergic terminals, whereas the measure of dopaminergic nigral neuron employed in our study may reflect an underlying neurodegenerative process in this area. All together results support the concept that $A_{2A}$ receptors display complex actions related to

the duration of insult, cellular elements and brain areas targeted by neurodegenerative processes.

Lack of a glial reaction in fbn$A_{2A}$KO mice, as compared to the robust astroglia and microglia activation in MPTP-treated control mice, indicates that deletion of neuronal $A_{2A}$ receptors may indirectly inhibit the inflammatory response. Glutamate is a main contributor to the complex neuron-glia cross-talk engaged by pathological events, which trigger both microglia and astroglia activation. For instance, by an action on NMDA receptors, glutamate release stimulates mitogen-activated protein kinases (MAPKs). Neuronal as well as glial p38 MAPK activation has been involved in cell suffering and apoptotic death, being activated and inducing several inflammatory mediators (Gianfriddo et al., 2004; Irving et al., 2000; Kawasaki et al., 1997; Piao et al., 2003). Therefore, $A_{2A}$ antagonists indirectly through a reduction of glutamate release might counteract glial reactivity and neuroinflammation in both SNc and Str (Melani et al., 2003, 2006). Activated glial cells, by the release of several toxic species as cytokines, free radicals, glutamate, is known to contribute to neuronal damage, and has been suggested to sustain a self-amplifying cycle which perpetuates MPTP toxicity (Hunot and Hirsh, 2003). Hence, interruption of such a detrimental vicious cycle might indirectly contribute to $A_{2A}$ receptor-dependent neuroprotection. Accordingly results suggest that in our model, operational glial $A_{2A}$ receptors did not contribute to MPTP-toxicity when $A_{2A}$ forebrain neuronal receptors where deleted. All together, our results suggest that $A_{2A}$ antagonists, by blocking neuronal $A_{2A}$ receptors, might act upstream in the cascade of toxic events and lead to an attenuation of dopamine neuron degeneration in PD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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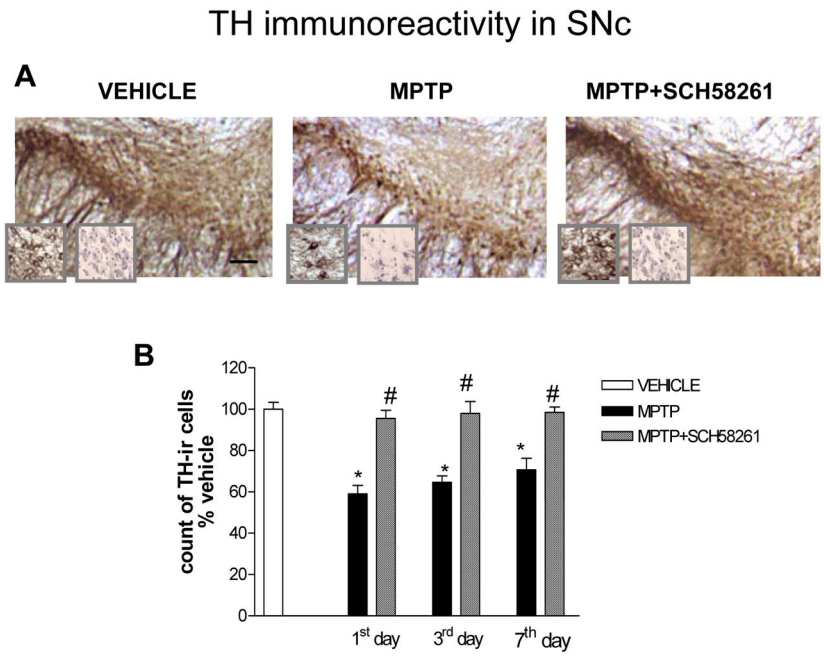


**Fig 1. Adenosine $A_{2A}$ receptor antagonist SCH58261 prevents dopaminergic cell loss in the SNc**

(A) shows representative sections immunostained for TH from SNc of mice sacrificed 3 days after MPTP treatment. Left insert shows TH-positive cells at higher magnification, right insert shows cresyl violet-stained sections; scale bar: 50 μm. Mice were treated with MPTP-HCl (20 mg/kg once a day for 4 days), plus SCH58261 (0.5 mg/kg) or vehicle (twice a day during MPTP treatment and once daily thereafter until sacrifice), and sacrificed 1, 3, 7 days after MPTP treatment. (B) shows analysis of TH immunostaining at 1, 3, 7 days after MPTP, reported as a percentage of TH-positive cells as compared to vehicle-treated mice. * indicates $p<0.001$ versus vehicle; # indicates $p<0.001$ versus MPTP group, by Tukey's *post hoc* test. Scale bar: 50 μm.

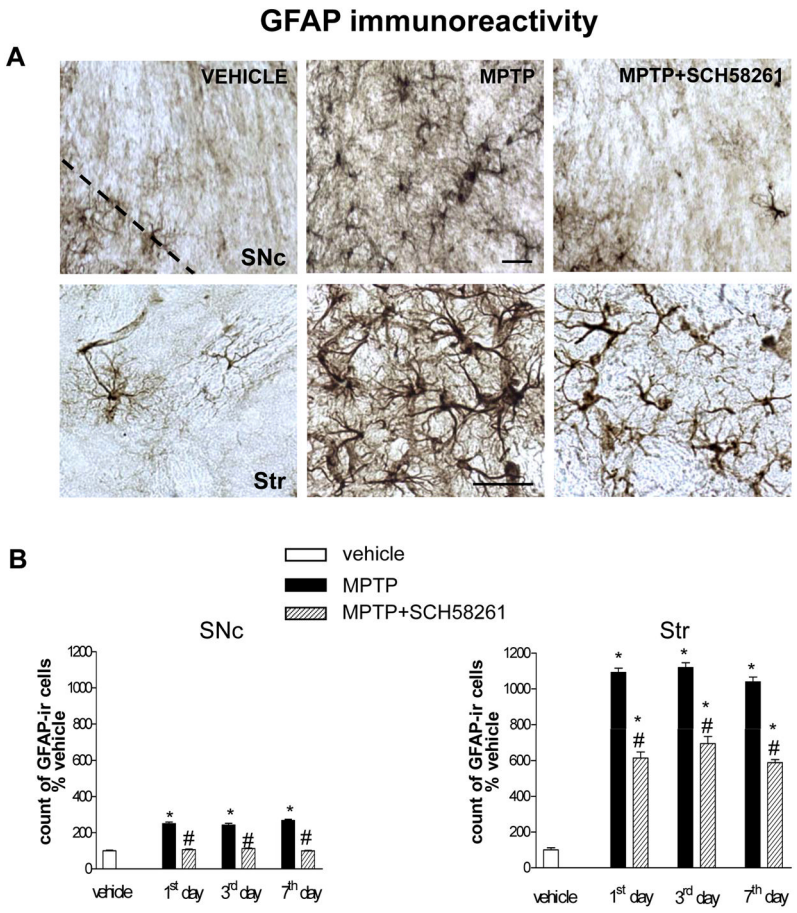


**Fig 2. Adenosine $A_{2A}$ receptor antagonist SCH58261 attenuates degeneration of dopaminergic terminals in the Str**

(A) representative sections immunostained for TH, from the Str of mice sacrificed 3 days after MPTP treatment. (B) Results from pharmacological blockade with SCH58261 or genetic $A_{2A}R$ depletion are presented. In the left column, − and + indicate the administration of vehicle or SCH58261to MPTP-treated mice. In the right column + and − indicate fbn$A_{2A}$WT and fbn$A_{2A}$KO mice, respectively.

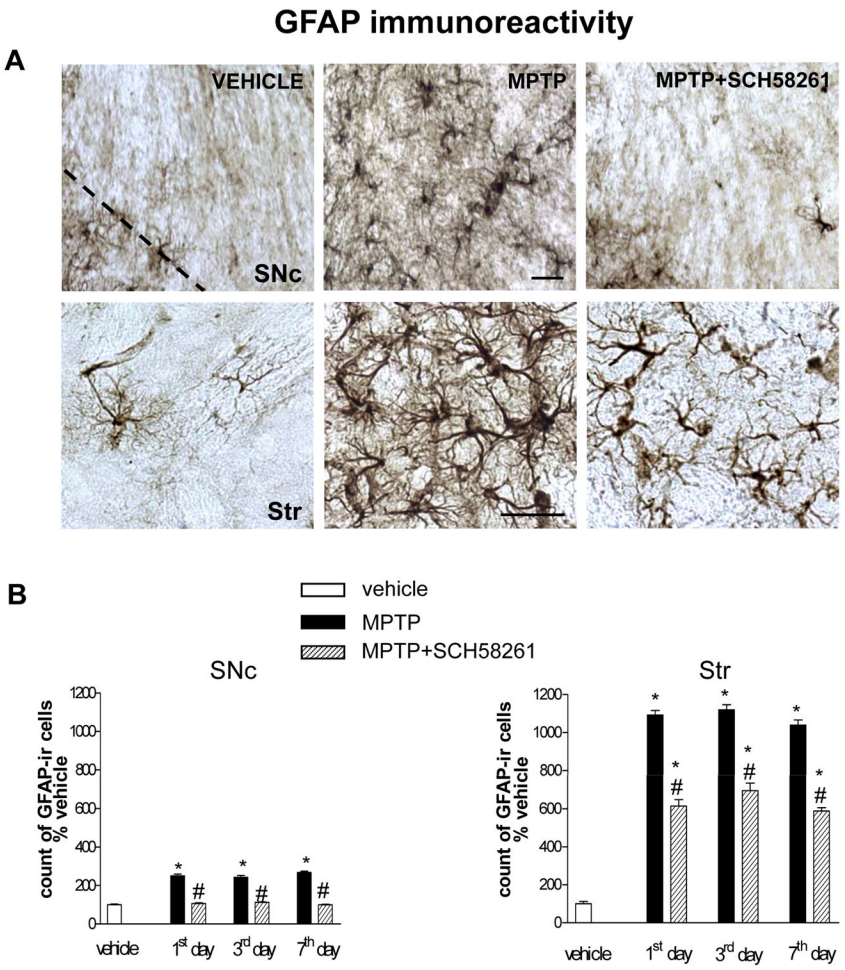


**Fig 3. Adenosine $A_{2A}$ receptor antagonist SCH58261 counteracts astroglia activation in the SNc and Str**

(A) shows representative sections immunostained for GFAP, from SNc (upper images) and Str (lower images) of mice sacrificed 3 days after MPTP treatment. Mice were treated as described in Fig 1. (B) shows analysis of GFAP immunostaining 1, 3, 7 days after MPTP, reported as percentage of GFAP-positive cells as compared to vehicle-treated mice in the SNc (left graph) and in the Str (right graph). * indicates $p<0.001$ versus corresponding vehicle and MPTP+SCH58261 groups; # indicates $p<0.001$ versus corresponding MPTP group, by Tukey's *post hoc* test. Scale bar: 50 μm.

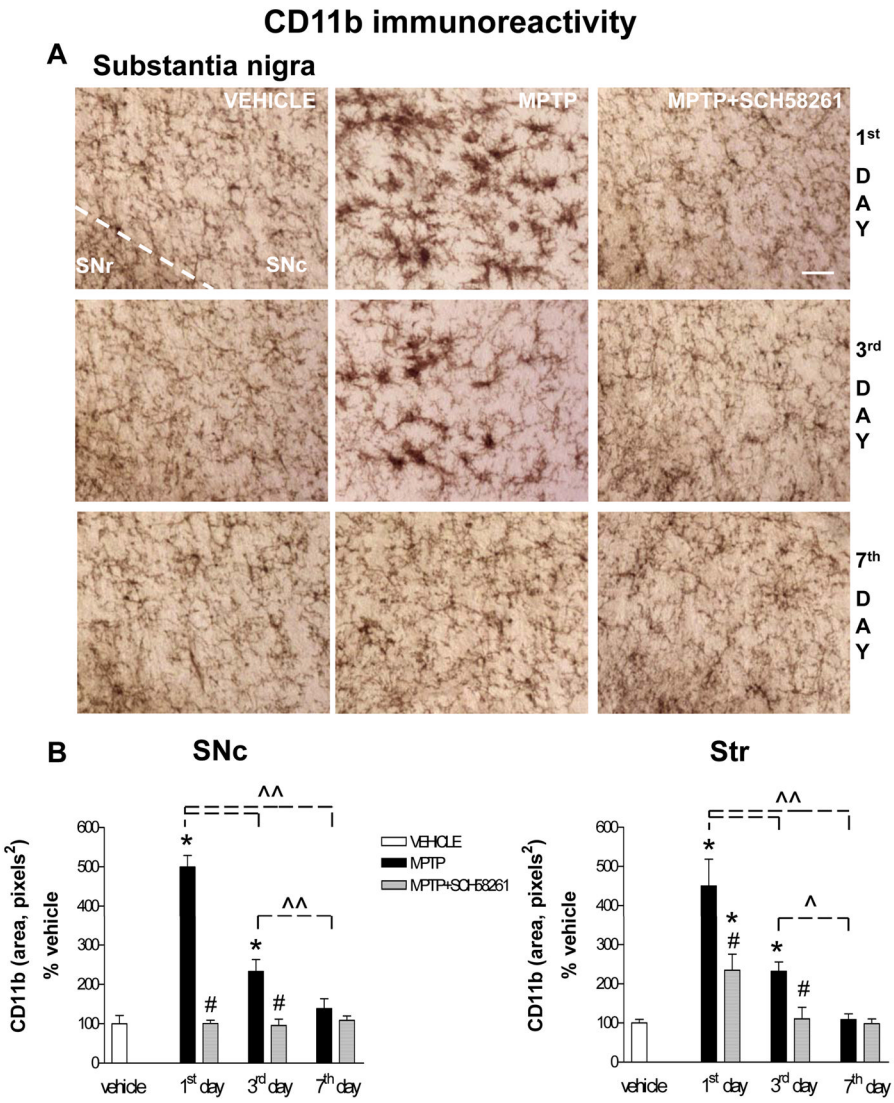


**Fig 4. Adenosine $A_{2A}$ receptor antagonist SCH58261 counteracts microglia activation**

(A) Representative images from the SNc immunostained for CD11b as a marker of microglia activation. Mice were treated as described in Fig 1 and sacrificed 1, 3, 7 days after MPTP treatment. (B) CD11b analysis in SNc and Str was performed in grey-scale digitized images. The area occupied by grey values above a threshold was calculated and expressed as square pixels and as percentage of vehicle-treated mice. Tukey's post hoc test: * $p<0.001$ versus vehicle and MPTP+SCH58261 group; # $p<0.001$ versus MPTP group; ^, ^^ $p<0.05$, $p<0.001$ versus the indicated time point. Scale bar: 50 μm.

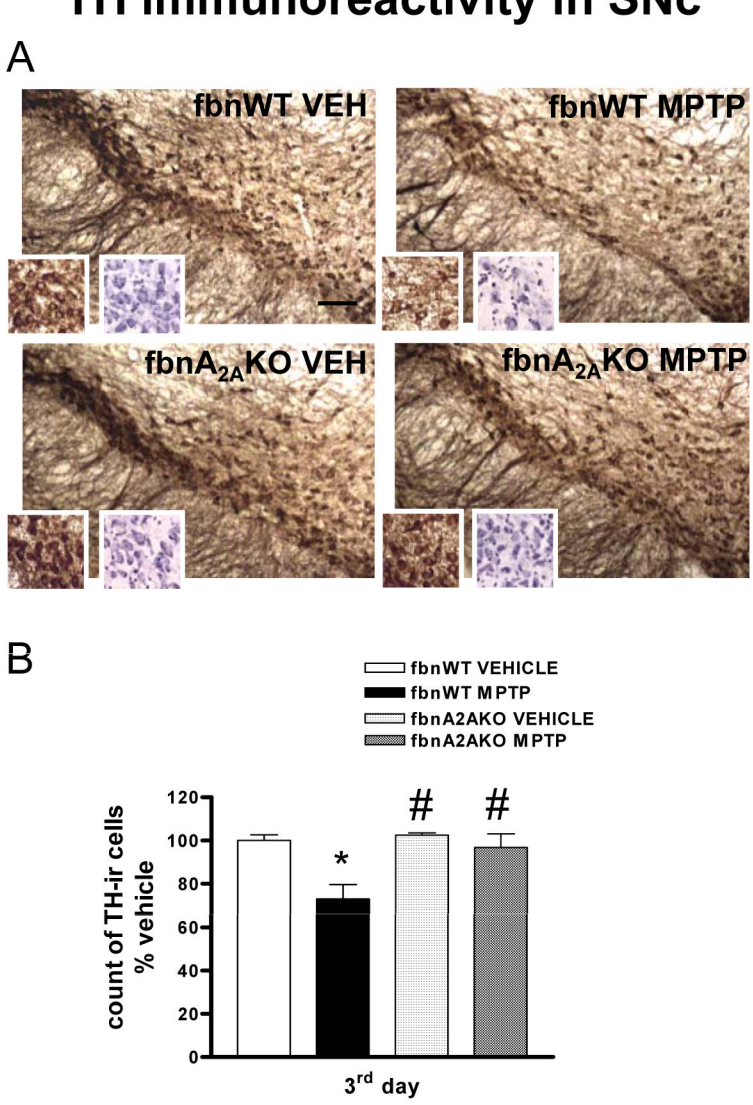


**Fig 5. fbn$A_{2A}$ KO mice are protected against MPTP-induced loss of dopaminergic cells in the SNc**

(A) shows representative sections from SNc immunostained for TH. Inserts show higher magnification of TH-labelled (left) and cresyl violet-labelled (right) cells. Mice were treated with MPTP (20 mg/kg once a day for 4 days)or vehicle. (B) shows analysis of TH immunostaining in fbn$A_{2A}$KO mice, reported as a percentage of TH-positive cells as compared to vehicle-treated mice. Tukey's post hoc test: * $p<0.05$ versus vehicle group; # $p<0.05$ versus WT MPTP group. Scale bar: 50 μm.

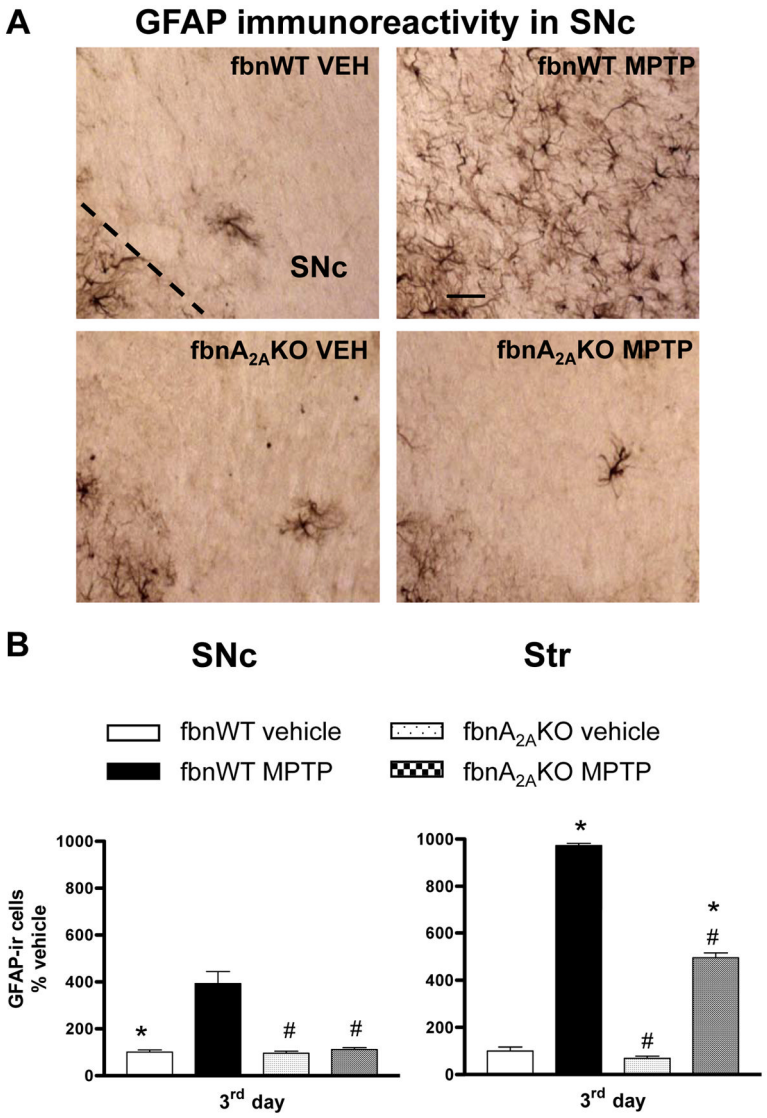


**Fig 6. Astroglia activation is attenuated in SNc and Str of fbn$A_{2A}$KO mice**

(A) representative images from the SNc immunostained for GFAP, as a marker of astroglial cells. (B) Graphs show the analysis of GFAP immunostaining in SNc and Str, in fbnWT and fbn$A_{2A}$KO mice treated with vehicle or MPTP. Tukey's post hoc test: * $p<0.001$ versus vehicle group; # $p<0.001$ versus WT MPTP group. Scale bar: 50 μm.

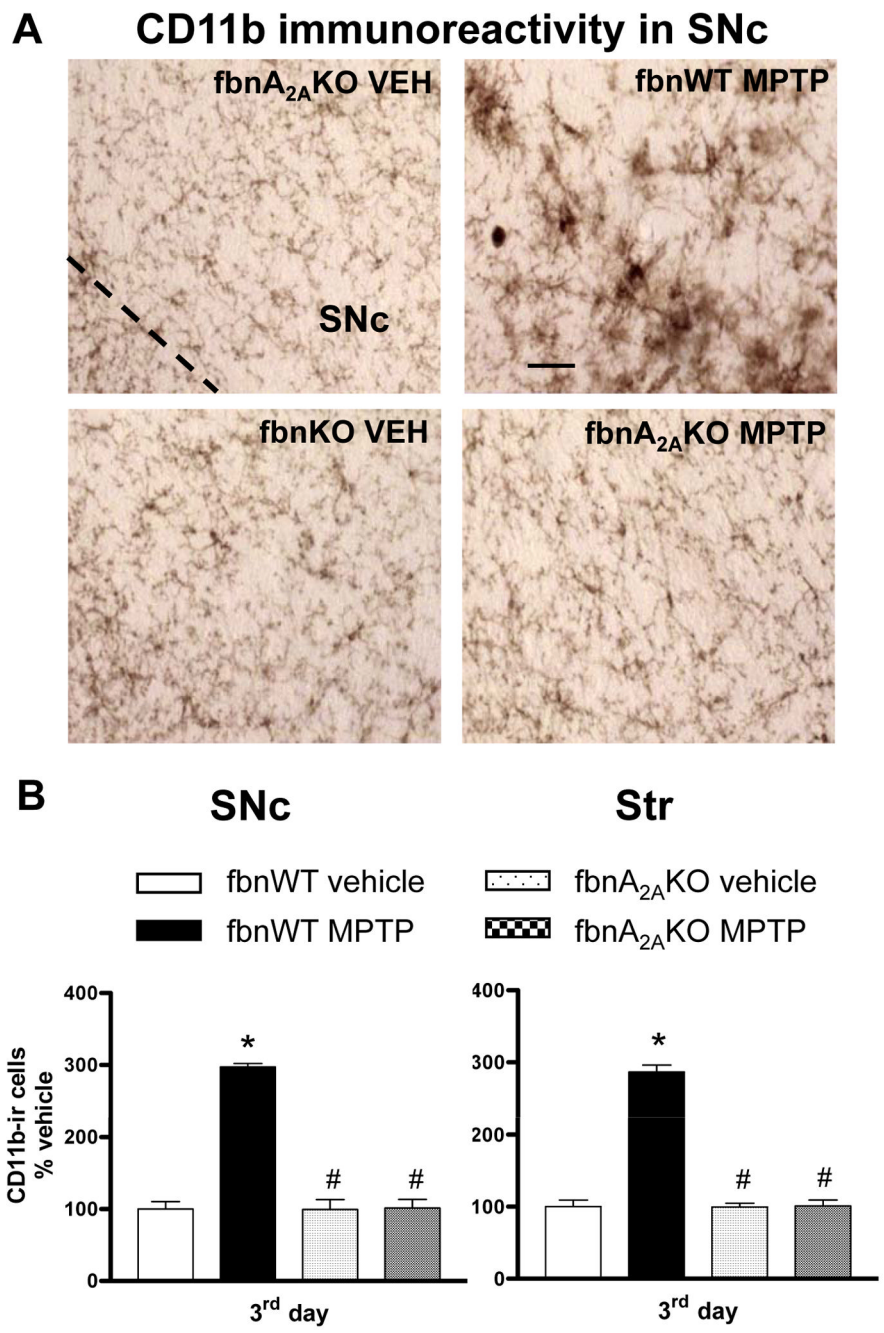


**Fig 7. Microglia activation is prevented in SNc and Str of fbn$A_{2A}$KO mice**

(A) representative images from the SNc immunostained for CD11b, as a marker of microglia activation. (B) Graphs show the analysis of CD11b immunostaining in SNc and Str, in fbnWT and fbn$A_{2A}$KO mice treated with vehicle or MPTP. Tukey's post hoc test: * $p<0.001$ versus vehicle group; # $p<0.001$ versus WT MPTP group. Scale bar: 50 μm.

**Table 1**

Unbiased evaluation of TH-IR and Nissl-stained neurons by stereological analysis in the SNc of mice following pharmacological blockade of $A_{2A}$Rs with the antagonist SCH58261 or $A_{2A}$R genetic depletion. Stereological evaluation of TH-IR and Nissl-stained neurons in the substantia nigra pars compacta

| Treatment | *N* | Density of TH-IR neurons/ $mm^3$ | Density of Nissl-IR neurons/ $mm^3$ |
|---|---|---|---|
| ***pharmacological blockade of $A_{2A}R$*** | | | |
| Vehicle | 3 | 29725.89 ± 439.56 | 37702.70 ± 2520,89 |
| MPTP | 3 | 15211.79 ± 1300.06* | 24827.52 ± 1689.72* |
| SCH + MPTP | 3 | 23003.66 ± 495.76*^ | 32817.67 ± 1225.20^ |
| ***fbn$A_{2A}R$ depletion*** | | | |
| WT vehicle | 3 | 27447.34 ± 1365.08 | 32198.28 ± 3341,77 |
| WT MPTP | 3 | 14278.85 ± 2493.30* | 21784.92 ± 2266.61* |
| KO vehicle | 3 | 23654.36 ± 2277.74 | 30903.52 ± 4264.73 |
| KO MPTP | 3 | 24267.96 ± 2277.83^ | 29832.48 ± 883.17^ |

* $p < 0.05$ versus vehicle group.

^ $p<0.05$ versus the corresponding MPTP treated group.

Appendix S

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# Alpha-Synuclein S129 Phosphorylation Mutants Do Not Alter Nigrostriatal Toxicity in a Rat Model of Parkinson Disease

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## Abstract

Lewy bodies are found in Parkinson disease and related disorders and are extensively phosphorylated at Ser-129, but whether S129 phosphorylation mediates ⟨-synuclein aggregation and neurotoxicity has been controversial. We used recombinant adeno-associated virus (rAAV) to overexpress ⟨-synuclein in the rat nigrostriatal system. Rats were injected with rAAV2/8 expressing either human wild type (wt) or mutant α-synuclein with S129 replaced by alanine (S129A) or aspartate (S129D). Contralateral substantia nigra injections containing empty vector served as controls. Both wt and S129 mutants resulted in significant dopaminergic cell loss in the recipients by 6 weeks but there were only small decreases in nigrostriatal terminal density and tyrosine hydroxylase (TH) expression. There were no significant differences in dopaminergic cell loss, nigrostriatal terminal density, or TH expression among the wt and S129 mutants. Furthermore, we did not observe any differences in ⟨-synuclein aggregate formation or distribution among wt and either S129 mutant. These findings contrast with those from previous studies and suggest that injections of both S129 phosphorylation mutants result in dopaminergic neurotoxicity similar to wt injections. Further study is needed to clarify the effects of these S129 mutants and α-synuclein phosphorylation in mammalian systems.

### Keywords

Adeno-associated virus; Dopamine; Lewy bodies; Striatum; Substantia nigra; Tyrosine hydroxylase

## INTRODUCTION

Parkinson disease (PD) is the most common neurodegenerative movement disorder and affects about 1% of the population over the age 65. The pathological hallmarks of PD are progressive loss of nigrostriatal dopamine neurons, swollen dystrophic neurites and characteristic filamentous intracellular inclusions called Lewy bodies. These inclusions are not unique to PD as they are also found in related disorders such as dementia with Lewy bodies and multiple system atrophy (1–3). A principal component of Lewy bodies is α-synuclein, a soluble, 140-amino acid protein that is abundantly expressed in the brain and is enriched in synaptic terminals (4). Although its normal function remains unclear, several point mutations in the α-synuclein gene (*PARK1/SNCA*) have been identified and linked to families with autosomal

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dominant parkinsonism (5–7). Gene duplication and triplication have also been linked to familial parkinsonism and a gene-dosage effect correlates with earlier disease onset and symptom severity (8–10). The fact that Lewy bodies are also found in these familial forms of parkinsonism further supports a role for α-synuclein in PD pathogenesis (11).

Abnormal folding, aggregation, and deposition of α-synuclein may be key steps in the pathogenesis of sporadic and familial PD (12). α-Synuclein self-aggregates and fibrillizes in vitro forming aggregates suggestive of those seen in Lewy bodies and Lewy neurites (13,14). Moreover, missense mutations in α-synuclein also result in accelerated fibril formation (15, 16) and oligomerization (17). Dose increases (i.e. duplication and triplication) of the α-synuclein gene promote this aggregation and deposition in insoluble fractions, consistent with inclusion formation (18). Animal models of human α-synuclein overexpression likewise demonstrate increased aggregates and neurotoxicity and recapitulate features of PD (19–21).

Epigenetic factors such as phosphorylation also influence α-synuclein fibrillization and aggregate formation. For example, the C-terminal portion of α-synuclein contains several phosphorylation sites (22,23) and there is evidence that Ser-129 phosphorylation accelerates α-synuclein fibrillization and enhances neurotoxicity. S129 is extensively phosphorylated in brains from patients with both sporadic and familial PD (24,25), as well as dementia with Lewy bodies (26). Although a small proportion (less than 5%) of α-synuclein is normally phosphorylated, nearly 90% of α-synuclein in Lewy bodies is phosphorylated at S129. Furthermore, *in vitro* S129 phosphorylation enhances fibril formation (24). In transgenic flies expressing human α-synuclein with mutation of S129 to either alanine (S129A) to prevent phosphorylation or aspartate (S129D) to mimic phosphorylation, blocking S129 phosphorylation suppresses dopamine neuronal loss, whereas S129D enhances α-synuclein neurotoxicity (27). In addition, overexpression of the *Drosophila* G-protein-coupled receptor kinase 2 (GRK2), which selectively phosphorylates α-synuclein at S129, results in enhanced neurotoxicity. Blockade of S129 phosphorylation (S129A) also increases inclusion formation and correlates with decreased α-synuclein neurotoxicity, suggesting that aggregates may be neuroprotective and sequester potentially toxic α-synuclein species. However, a recent study in rats found the opposite and demonstrated that the S129A mutant was more toxic than wild-type human α-synuclein and associated with decreased intracellular aggregates, whereas the phosphomimic S129D appeared to be neuroprotective (28). Thus, whether or not phosphorylation at S129 plays a critical role in mediating α-synuclein-induced dopaminergic neurotoxicity remains uncertain.

In this study we examine the effect of α-synuclein S129 phosphorylation state on dopaminergic neurotoxicity in a rat model of PD. Previous studies have established that viral-vector based overexpression (using either recombinant adeno-associated virus [rAAV] or lentivirus) of human α-synuclein in the rat nigrostriatal system results in progressive dopaminergic cell loss, synuclein-rich aggregates, and dystrophic neurites (29,30). New rAAV serotypes are increasingly being used and they show greater transduction efficiency and delivery of transgene into the nervous system compared to the commonly used rAAV2/2 (31–33). Here, we present new data using rAAV2/8 for delivery of α-synuclein S129 phosphorylation mutants; we find no significant differences in nigrostriatal dopaminergic toxicity or morphology of α-synuclein aggregates among wild type (wt) and S129A/D mutants.

## MATERIALS AND METHODS

### Virus Production

Construction of rAAV vectors used to express human wild-type α-synuclein was as previously described (34). Overlap extension PCR was used to generate α-synuclein S129 mutants (S129A and S129D) using the mammalian pcDNA-synuclein (human wt, GenBank L08850) vector as

template and previously designed primers (available by request). S129 mutant and wt α-synuclein were then cloned into AAV-CBA-WPRE vector using the subclone internal ribosomal entry site enhanced green fluorescence protein (pIRES2-EGFP) (Clontech, Mountain View, CA). We also constructed an empty vector, AAV-CBA-WPRE, with no transgene (kindly provided by Dr. Miguel Sena-Esteves, Massachusetts General Hospital [MGH], Boston, MA). Recombinant AAV2/8 virus was generated via tripartite transfection of the *cis*-transgene, packaging (*rep* and *cap*) genes, and helper plasmid into HEK 293A cells (Harvard Gene Therapy Initiative, Harvard Medical School). Viral particles were purified by iodixanol density gradient, isolated, and titered by dot blot hybridization. Final titers for virus were for α-synuclein wt $1.4 \times 10^{13}$ gc/mL, S129D $1.4 \times 10^{13}$ gc/mL, S129A $7.7 \times 10^{12}$ gc/mL, and empty vector $1.16 \times 10^{14}$ gc/mL.

## Stereotaxic Viral Injections

All animal protocols and procedures were approved by the MGH Subcommittee on Research Animal Care. The nigrostriatal system is essentially unilateral, thus each animal received bilateral stereotaxic injections of rAAV into the substantia nigra (SN). In most animals, virus with empty vector was injected contralateral to virus encoding human wt or mutant S129A/D α-synuclein. Sprague Dawley rats (300–350 g) were anesthetized, placed in a Kopf stereotax, and bilateral small skull holes were drilled to expose the dura over the injection sites. Coordinates for SN injections from bregma were AP −5.2, ML ± 2.0, and DV −7.4 from the dural surface and targeted the central SN pars compacta (SNc). For each virus, $1.4 \times 10^{10}$ gc in 2 μL were injected at 0.2 μL/minute using a microinjection pump (Stoelting Co., Wood Dale, IL) with 10 μL Hamilton syringe and 33-gauge needle. After injection the syringe remained in situ for 5 minutes before withdrawal.

## Tissue Preparation and Immunohistochemistry

At 2 or 6 weeks post-injection, rats were deeply anesthetized and transcardially perfused with cold 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains from a subset of 6-week-old animals were removed without fixation, and the striatum and midbrain quickly dissected on ice and snap-frozen in isopentane for use in dopamine content measurement and immunoblotting. Perfused brains were postfixed for 24 hours, then cryoprotected in 30% sucrose/PBS, and serially sectioned at 40 μm on a sliding microtome into 12 wells (forebrain and midbrain separately blocked). Sections were collected and stored in cryoprotectant, 30% sucrose and 30% ethylene glycol in PBS, until processed and analyzed. Briefly, free-floating sections were rinsed with PBS, then treated for 3 to 5 minutes with 10% methanol and 3% $H_2O_2$ to inhibit endogenous peroxidases, permeablized with 0.3% Triton X-100 in PBS, and blocked in 5% normal goat serum. Coronal sections through the striatum and nigra were immunostained with primary antibodies to tyrosine hydroxylase ([TH] 1:10,000 dilution; Millipore, Billerica, MA) or α-synuclein LB509 (1:1000 dilution; Zymed Laboratories, Inc., San Francisco, CA) overnight at 4°C. After washing, immunostaining was visualized with either fluorescent secondary (1:200 dilution; Alexa Fluor 488, Molecular Probes, Eugene, OR; or Cy3, Jackson ImmunoResearch, West Grove, PA) or biotinylated secondary, followed by avidin-biotin (Vectastain Elite Kit, Vector Labs, Burlingame, CA), and 3,3'-diaminobenzidine reaction. Immunostained sections were washed, mounted on Superfrost slides, and coverslipped (GVA mount, Zymed Laboratories, Inc., San Francisco, CA; or Permount, Sigma Chemicals, St. Louis, MO). In some animals, adjacent coronal sections were also Nissl-stained with 0.05% cresyl violet per standard protocols.

## Microscopy and Stereology

Immunostained sections were viewed using an Olympus BX51 microscope with bright-field and epifluorescence attachment. Photomicrographs were taken with an Olympus DP70 digital

camera and adjusted only for suitable contrast and brightness. To maintain detail, wide-field images were photo-montaged from high-power photos using Adobe Photoshop CS3. Double-label immunofluorescence images of cells were obtained using a Zeiss LSM510 confocal microscope system. Images were obtained with multi-tracking to minimize spectral overlap.

Nigrostriatal cell loss was assessed by counting TH-immunoreactive cells in the SNc, including the adjacent pars lateralis and caudal dense cell group ventral to the medial lemniscus and retrorubral area, using unbiased stereology according to the optical fractionator principle (35). At least 8 sections (each 240 μm apart) through the SN for each animal were analyzed and counted using the Olympus CAST Stereology System. Sampling intensity was sufficient for a coefficient of error for the counting protocol of less than 0.1.

## Striatal TH Terminal Expression

Semiquantitative analysis of TH expression in nigrostriatal terminals at 6 weeks post-injection virus was performed by measurement of optical density of immunofluorescence intensity scans using a Scan Array Express (Perkin Elmer, Boston, MA). All sections for analysis were handled, washed, and immunostained at the same time, using common reagents to minimize inter-animal variability. Striatal sections approximately 1 mm rostral to the decussation of the anterior commissure were immunostained with antibody to TH and fluorescent secondary-Cy3 (Molecular Probes), and scanned using 543-laser excitation. Using ImageJ (NIH), mean gray value of fluorescence intensity within the dorsolateral striatum was obtained for each section and normalized to background, corpus callosum.

## Immunoblotting

Striatal and midbrain tissue were separately suspended in 300 μL lysis buffer (50 mM Tris-HCL, pH 7.4; 175 mM NaCl; 5M EDTA, pH 8.0; and protease inhibitor [Roche]) and homogenized on ice for 10 to 15 seconds. Each sample was centrifuged for 15 minutes at 4° C, filtered, and then 1% Triton X-100 added to the lysate. After 1 hour incubation on ice, lysates were centrifuged for 60 minutes at 4°C to collect the triton-X insoluble fraction. Triton-soluble lysate was separated and the insoluble pellet resuspended in 2% SDS-containing lysis buffer (Triton-insoluble fraction), then sonicated for 10 seconds. Protein concentration for each lysate was determined by bicinchoninic acid (BCA) assay. Five μg of each sample were separated on 10% to 20% Trisglycine pre-cast gel (Invitrogen, San Diego, CA), transferred to polyvinylidene diflouride (PVDF), and immunoblotted for TH (mouse TH-2 antibody, Sigma) or α-synuclein (mouse Syn1, BD Transduction Laboratories, or Syn (LB509), Zymed, antibody). All blots were immunostained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit antibody, Abcam, Cambridge, MA) as loading control. In some cases samples for each condition were pooled. Immunoblotted α-synuclein, TH and GAPDH were detected with secondary antibody conjugated to horseradish peroxidase (HRP) and reacted with ECL (GE Healthcare, Waukesha, WI), per protocol. Films were scanned using a FluorChem system and analyzed with ImageJ software (NIH). TH and α-synuclein content for each sample was normalized to GAPDH.

## Dopamine Content

Striatal tissue was thawed, homogenized, and mixed in lysis buffer with dihydrobenzylamine added as internal control. Dopamine and 3,4-dihydroxyphenlyacetic acid (DOPAC) were measured by high performance liquid chromatography (HPLC) with electrochemical detection and normalized to protein content per sample (36).

### Statistics

All data were expressed as group mean ± SEM. Stereological estimates of nigral TH cell survival, mean striatal terminal density, and dopamine measurements were analyzed using one-way ANOVA with Bonferonni's multiple comparison post-hoc (Prism GraphPad 4.03, San Diego, CA) unless otherwise stated.

## RESULTS

We used the pseudotyped rAAV 2/8 for delivery of transgene because recent rAAV serotype comparisons have shown increased transduction efficiency over that of rAAV2/2 (33,36). Transgene expression for rAAV2/8 is also markedly enhanced over rAAV2/2 and rapidly reaches a steady state by 2 to 4 weeks (37,38). Rat SN were stereotaxically injected with equal volume and genome copies of rAAV vector encoding either human wt or S129 phosphorylation mutant (S129A for substitution to alanine, or S129D for aspartate) α-synuclein, followed by an internal ribosomal entry site (IRES) and the reporter gene EGFP, resulting in bicistronic expression. To control for possible viral toxicity and injection effects, the contralateral SN of several rats was injected with empty rAAV vector and used as an internal control. Based on preliminary experiments with rAAV2/8, its rapid transgene expression and spread, we selected 2-and 6-week time points post-injection for analysis of wt and S129A/D α-synuclein toxicity in the nigrostriatal system.

The patterns of transgene expression for all rAAV were similar. For α-synuclein viruses, EGFP expression marked cells transduced with rAAV in the SN. All cells with EGFP co-expressed α-synuclein as assessed by double immunofluorescence. rAAV2/8 was neurotrophic; we did not observe transduction of glial elements. At 2 and 6 weeks post-injection, EGFP expression was widespread for α-synuclein rAAV and included cells not only in the SNc, but also in the adjacent pars reticulata (SNr), ventral tegmental nucleus, and a portion of the ventral mesencephalic reticular formation (Fig. 1). These findings are consistent with the reported enhanced transduction efficiency of rAAV2/8 compared to rAAV2/2 in the SN (33,34,37).

In addition to cellular expression of α-synuclein within the SN, immunostaining for human α-synuclein using the LB509 antibody (specific for amino acid residues 115–122) also demonstrated extensive transport of α-synuclein to nigrostriatal terminals (Fig. 2). Animals without extensive nigrostriatal labeling suggested failed or misplaced injections and were excluded from the analysis. Synuclein-positive terminals were widespread and found throughout the dorsal striatum (caudatoputamen), extending rostrally to the striatal pole and several millimeters caudal to the anterior commissure. These findings are consistent with the topographic distribution of nigrostriatal projections (39) and confirm transduction of a large proportion of SNc neurons for each case. Double labeling of SN with antibodies to α-synuclein (or GFP) and TH also demonstrated that rAAV injections transduced a majority of dopaminergic nigrostriatal neurons in the SNc and adjacent regions (data not shown).

### Viral Expression

To assess rAAV α-synuclein expression levels for wt, S129A and S129D mutants, we performed Western blot analyses on striatal and midbrain extracts from 3 animals per group obtained at 6 weeks post-injection and compared these to extracts from the contralateral hemisphere injected with empty vector control. Blots from samples taken from wt, S129A, and S129D α-synuclein recipients probed with the LB509 synuclein antibody demonstrated strong human α-synuclein expression ipsilateral to the injection, as expected. We also probed blots with the Syn-1 antibody (BD Transduction, San Jose, CA), which recognizes both endogenous rat and human α-synuclein, to estimate the level of human α-synuclein overexpression for each rAAV compared to endogenous levels (Fig. 3). Comparison of the α-synuclein injected side

to the contralateral striatum or midbrain, empty rAAV vector control, revealed levels of α-synuclein overexpression with a mean ratio (ipsilateral/contralateral side) of approximately twice that of endogenous rat α-synuclein.

## Comparison of Nigrostriatal Toxicity

Unbiased stereological estimates of TH-positive cells in the SN were performed at 2 and 6 weeks post-injection of rAAV expressing wt or mutant, S129A or S129D, α-synuclein and compared to empty vector injections. In preliminary studies, subtle TH cell loss was observed at 2 weeks in both wt and mutant S129A and S129D groups (n = 2 to 3 for each group). These findings were somewhat surprising given the early time point, but dopaminergic neurotoxicity for S129A has been observed as early as 4 weeks (28). Qualitative comparison of TH cell loss with cresyl violet-stained sections confirmed proportional nigral cell loss and gliosis. Given the modest extent of cell loss at 2 weeks, we examined a larger cohort (6 to 10 per group) at 6 weeks to further compare toxicity of wt and S129 mutant α-synucleins.

At 6 weeks post-injection there was TH cell loss evident in the SNc for both wt and S129 mutant α-synuclein groups (Fig. 4). Cell loss, as opposed to TH-phenotype loss, was confirmed by analysis of Nissl-stained sections and qualitative comparison with nigral TH cell loss. Stereological estimates of remaining TH cells in the SN demonstrated significant cell loss compared to empty vector control for wt (20.5 ± 6.8%, $p < 0.05$), S129A (29.9 ± 2.4%, $p < 0.001$), and S129D (27.0 ± 6.7%, $p < 0.01$) mutants ($F[3,24] = 9.96$, $p = 0.0002$) (Fig. 5A). The mean TH cell loss was 25.8% and ranged from 7% to 45% for all animals analyzed. Post-hoc analysis revealed no significant differences in TH cell counts between wt, S129A, or S129D. Qualitative comparison of nigral TH staining at 6 weeks also did not differ.

## Nigrostriatal TH and Dopamine Content

Immunostaining of striatal sections for TH did not show nigrostriatal terminal loss at 2 weeks. By contrast, at 6 weeks TH-immunostaining revealed loss of terminal density in the dorsal striatum for both S129A and S129D compared to wt recipients (Fig. 2). Semiquantitative analysis, using scan array measurement of immunofluorescence optical density also revealed small decreases relative to control in dorsal striatal TH-positive terminal density for wt and mutant S129A and S129D recipients, but these findings did not achieve statistical significance (Fig. 5B). Relative side-to-side comparisons of recipients with internal empty vector control showed a similar trend of decreased dorsal striatal TH-positive terminal density for S129A and S129D mutant animals but also did not achieve significance.

To investigate potential changes in striatal dopaminergic innervation further, we also quantified striatal dopamine content at 6 weeks post-injection via HPLC fractionation and electrochemical detection. Dopamine was extracted from striatal tissue ipsilateral to the SN injection of either wt or mutant (S129A or S129D) α-synuclein and compared to that extracted from the contralateral control striatum (SN injected with empty rAAV vector). In wt recipients, the striatal dopamine content and levels of its metabolite, DOPAC, did not differ (Fig. 5C). In contrast, relative dopamine content for S129A did significantly decrease, mean ratio (ipsilateral/contralateral striatum) 0.66 ± 0.04 ($p = 0.006$, one sample *t*-test for hypothesized ratio of 1). Dopamine levels also decreased for S129D (mean ratio 0.59 ± 0.15), but did not achieve statistical significance ($p = 0.055$). Similarly, striatal DOPAC content for both S129A and S129D recipients demonstrated a trend toward decrease but were not significant. These results are consistent with the loss of 25% of TH-positive neurons in the SN.

## Synuclein Toxicity, Cellular Aggregates, and Terminal Morphology

Examination of sections stained for human α-synuclein (LB509) at 2 and 6 weeks using bright-field and confocal microscopy revealed progressive changes in nigral cell morphology.

Synuclein-positive cells in wt and mutant S129A and S129D cases were densely immunostained throughout the nucleus and cytoplasm (Fig. 6). A subset of cells contained synuclein-positive aggregates at 6 weeks. Some larger cell aggregates co-stained for α-synuclein and ubiquitin. There were no qualitative differences in aggregate size or distribution between wt and S129A or S129A cases. Transduced cells frequently had dystrophic process as previously described (29). Examination of nigrostriatal terminals immunostained for human α-synuclein also showed dystrophic changes and neurites, but no qualitative differences were noted between wt, S129A or S129D animals.

## DISCUSSION

We examined the role of α-synuclein phosphorylation at Ser-129 by comparing the effects of overexpression of human wt and mutant α-synuclein S129A (alanine blocks phosphorylation) and S129D (mimics constitutive phosphorylation) in the rat nigrostriatal system. A targeted viral vector-based approach with rAAV2/8 was used to overexpress α-synuclein in the SNc. Neither Ser-129 α-synuclein mutant appeared to significantly alter dopaminergic nigrostriatal toxicity in the rat PD model. At 2 weeks, early neurodegenerative changes and TH cell loss were apparent, and by 6 weeks overexpression of wt and S129A and S129D mutants resulted in significant (20%–30%) nigral dopaminergic cell loss (confirmed on Nissl-stained sections) compared to empty vector control. There were, however, no significant differences in dopaminergic nigrostriatal cell toxicity between wt, S129A, or S129D α-synuclein at 6 weeks. Furthermore, the size and distribution of α-synuclein aggregates did not differ among wt and either S129 mutant. Consistent with these findings, we did not detect major changes in striatal TH-terminal density or expression, although striatal dopamine content was decreased for S129A and S129D. The normal striatal dopamine levels for wt α-synuclein are not surprising because over 6 weeks non-geriatric rodents have the ability to recover dopamine content after nigrostriatal lesion such as that caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (40). The lack of recovery of dopamine content for both S129 mutants thus may suggest a decrease in functional neurochemical compensation or plasticity. Morphologically, α-synuclein-positive nigrostriatal terminals in S129 recipients did not differ significantly from those of wt but S129 mutants may alter dopamine release and synaptic function since these are putatively regulated by α-synuclein (41).

These findings contrast with those from previous studies in similar *Drosophila* and rat PD models that also compared wt α-synuclein and S129A and S129D phosphorylation mutants (27,28). In the fly, S129D was more toxic to dopamine cells and S129A was not only protective, but also associated with an increase in α-synuclein aggregates. Recent data in rats found the opposite result and demonstrated increased toxicity for S129A and possible neuroprotective effects for S129D (28). Toxicity was, however, similarly associated with a decrease in large, insoluble α-synuclein aggregates whereas neuroprotection correlated with the presence of cytoplasmic inclusions. By contrast, we observed no differences in toxicity or aggregate formation for wt α-synuclein or the phosphorylation mutants S129A and S129D in the rat.

Differences in study design may in part explain these differing results among studies. We used rAAV2/8 to deliver and overexpress α-synuclein in the rat SN, whereas Gorbatyuk et al used a different serotype, rAAV2/5 (28). Both of these rAAVs show enhanced transduction efficiency (i.e. increase in cell number and volume transduced) over that of rAAV2/2 in the rodent nigrostriatal system (33,38,42). However, rAAV2/8 results in higher transgene expression than other rAAV serotypes, including 2/2 and 2/5 (37). One concern is that elevated levels of α-synuclein expression from high-titer AAV2/8 injections may mask differences in toxicity between wt and the S129 mutants. Therefore, we injected a moderate dose of each virus ($1.4 \times 10^{10}$ gc), resulting in only 20% to 30% nigral TH cell loss at 6 weeks. Using a similar dose of AAV2/5, Gorbatyuk et al reported 40% to 60% cell loss for both wt and S129A,

albeit at 8 weeks post-injection. Moreover, Western blot estimates of human α-synuclein expression compared to endogenous rat α-synuclein were also lower (only 2-fold) than that previously reported. These findings suggest that the amount of virus injected and, indirectly, the levels of α-synuclein expression in our study did not “saturate” the system. The fact that we saw toxicity in S129D recipients also argues that the viral dose was not too low, since previously it appeared to be less toxic than the wt (28).

Another difference between this and previous studies is the length of viral incubation. The time course for expression of the transgene is similar for both rAAV 2/5 and 2/8, although perhaps a bit more rapid for rAAV2/8 (38). The 2 and 6 week time points for analysis we chose may have been too early to detect differences in toxicity among wt and S129 mutants. Even at 4 weeks, however, toxicity was noted for S129A by Gorbatyuk et al and was evident by 2 weeks in this study (28). Moreover, by 6 weeks, the wt and both S129 mutant recipients showed significant dopaminergic cell toxicity.

Recent *in vitro* data on S129 phosphorylation and phosphomimics (e.g. S129D/E) may also help to explain differences in results from rat and fly PD models. S129 phosphorylation increases α-synuclein conformational flexibility and appears to inhibit fibril formation. Although they are designed as phosphomimics, S129D/E do not exactly reproduce the effects of native phosphorylation (43). S129 phosphomimics only demonstrate local changes in conformational dynamics and form fibrils similar to those formed by wt in vitro but not as rapidly as S129A. Consistent with these findings we found similar toxicity for wt and S129D α-synuclein. Rapid fibrillization due to S129A presumably leads to increased α-synuclein aggregate formation that is thought to sequester toxic species. This hypothesis is supported by reduced S129A toxicity and increased α-synuclein inclusions seen in the fly (27). Contradictory results for S129A are, however, seen in the rat (28), possibly suggesting that while fibrils form, the S129A mutant in a mammalian system may also enhance formation of toxic α-synuclein oligomers. Together, these findings emphasize the importance of phosphorylation at S129 as a regulator of α-synuclein function but do not fully clarify its effects on toxicity.

Increasing evidence suggests that large α-synuclein aggregates may be neuroprotective and that S129 phosphorylation plays a role in aggregate formation (44). A high proportion of α-synuclein in Lewy bodies is phosphorylated at S129 (24,25). Furthermore, co-expression of human α-synuclein and protein kinases, such as G-protein-receptor kinase 5 (GRK5)—one of many kinases that phosphorylate at S129 (27,45)—is associated with α-synuclein aggregate formation in cell culture (46). Recent data indicate, however, that S129 phosphorylation can also inhibit fibril formation and α-synuclein oligomerization but with longer incubation times fibrillary aggregates such as those found in Lewy bodies eventually form (43). Lingering questions remain as to what influences α-synuclein phosphorylation, when it occurs (before, during or after fibrillization and aggregate formation), and how it may mediate neurotoxicity.

Several kinases phosphorylate α-synuclein at S129, including casein kinases 1 and 2, and GRK2 and 5 (22,45,47), but specific phosphatases have yet to be identified. Regulation of the α-synuclein phosphorylation state in vivo thus remains unclear, in particular due to the multiple cellular compartments in which α-synuclein is found. Phosphorylation of α-synuclein by GRKs is enhanced by phospholipids (45). Liposomal binding of α-synuclein and GRK appears to increase its kinase activity (48). α-Synuclein phosphorylation by casein kinase 2 is similarly enhanced by liposomes. There is also evidence for $Ca^{2+}$/calmodulin interaction with GPK5 and activation of α-synuclein phosphorylation (49). Further study is necessary to identify other specific kinases and protein and membrane interactions involved in α-synuclein phosphorylation.

The function of α-synuclein remains largely unknown and further complicates our understanding of its role in PD pathophysiology. Putative functions include regulation of synaptic dopamine transport (41), vesicle formation and trafficking (51), mitochondrial function (41,50), and nuclear transcription (51). How α-synuclein phosphorylation affects these functions is unclear, but disruption in any one of these could lead to dysfunction and potential neurotoxicity. Findings presented here suggest that the phosphorylation state at S129 and its effects *in vivo* are more complex than previously thought and that it may not play a critical role in mediating dopaminergic toxicity. A better understanding of α-synuclein phosphorylation and aggregate formation is clearly needed and will help target novel therapies.

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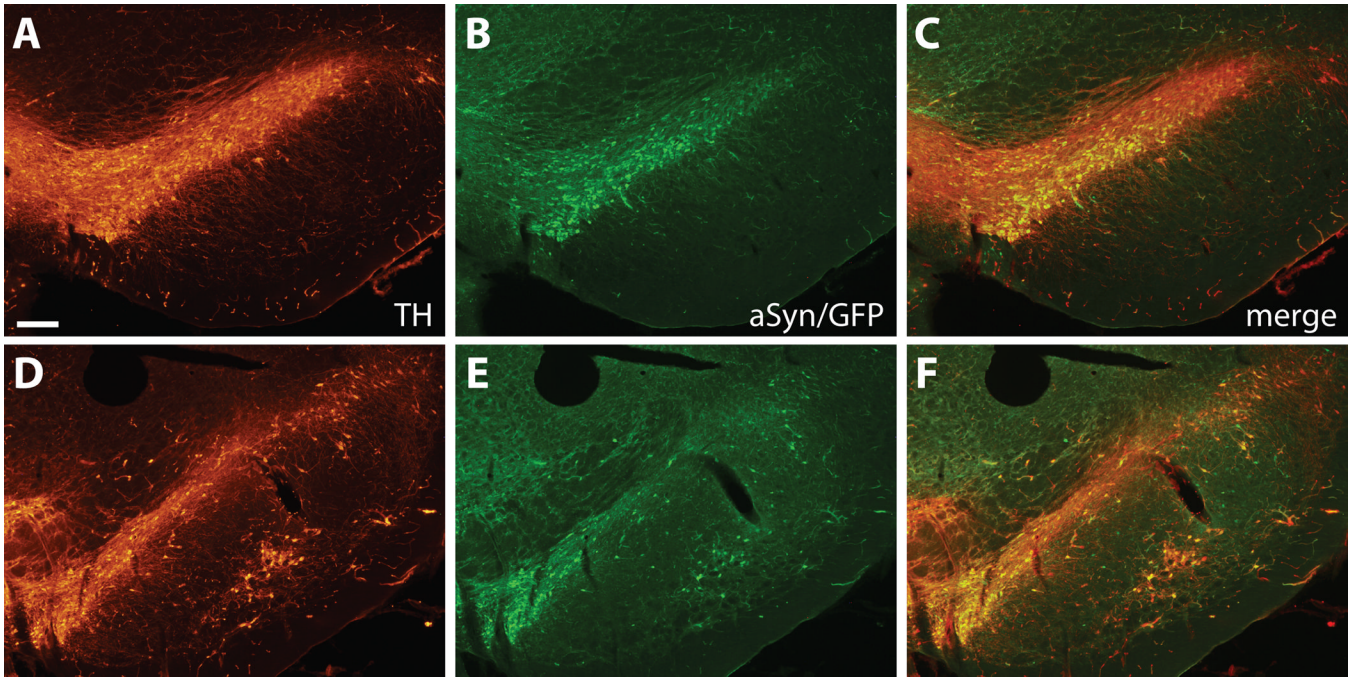
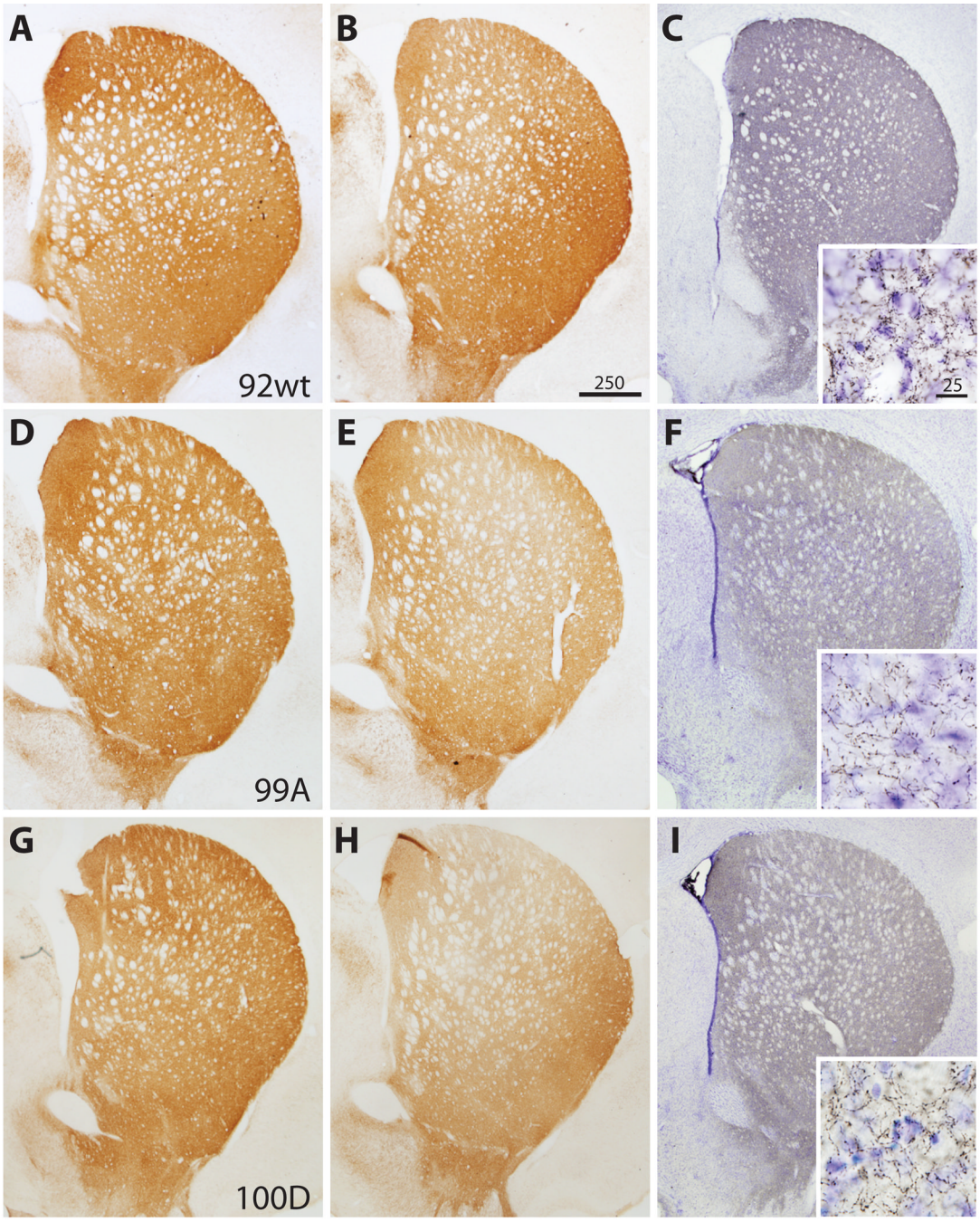



**Figure 1.**

Immunofluorescence staining of substantia nigra (SN) injected with rAAV2/8 expressing wt α-synuclein and internal ribosomal entry site-enhanced green fluorescent protein (EGFP). Tyrosine hydroxylase (TH) staining shows the distribution of dopaminergic SN neurons at representative rostral (**A–C**) and caudal (**D–F**) levels. GFP-positive transduced cells are widely distributed throughout the SN and almost exclusively colocalize with TH-positive neurons. Bar: 250 μm.



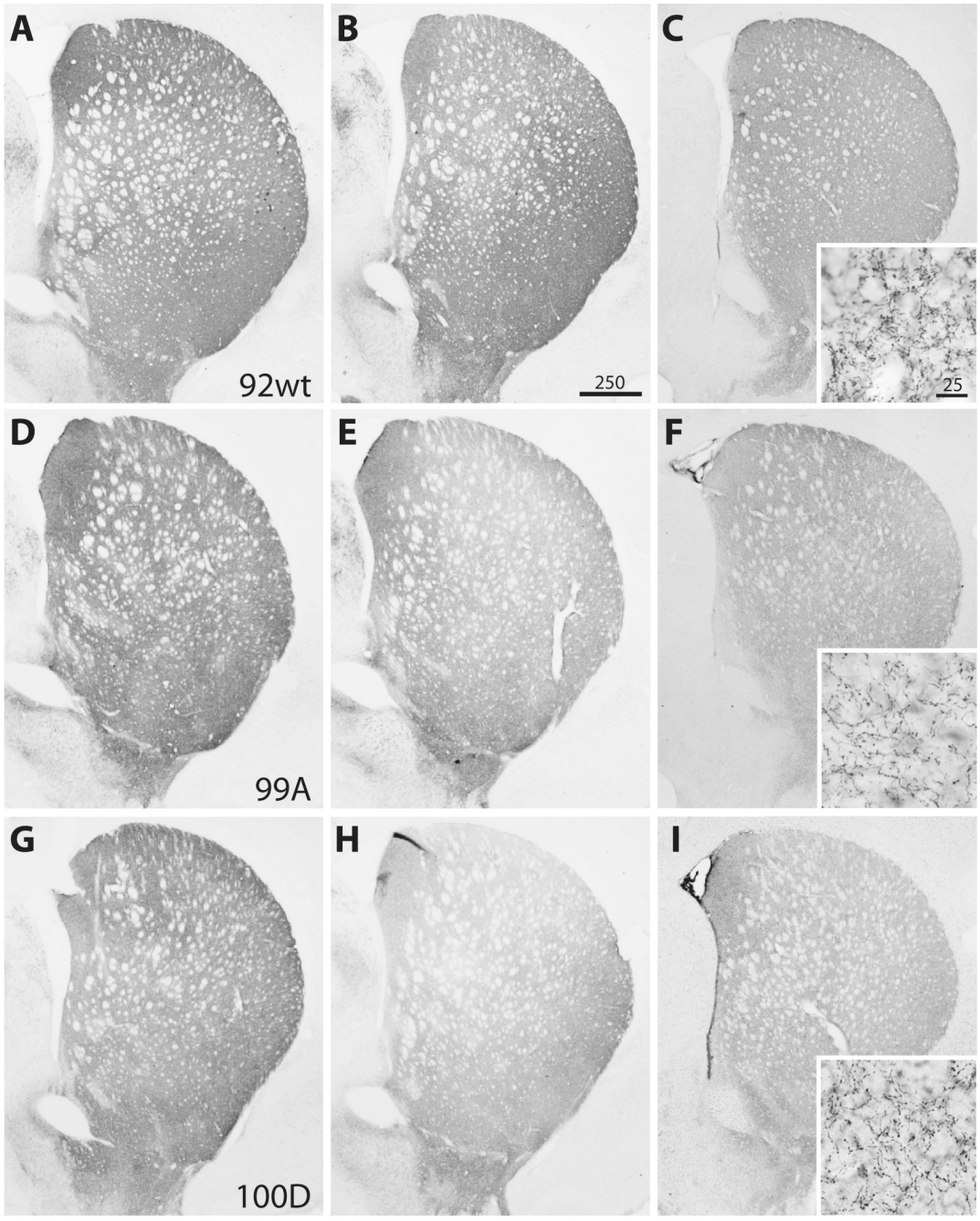



**Figure 2.**

The distribution of nigrostriatal terminals immunostained for tyrosine hydroxylase (TH) and human α-synuclein (LB509) at 6 weeks post-injection. Columns 1 and 2 compare striatal TH terminal labeling after empty vector control (**A, D, G**) and ⟨-synuclein (**B, E, H**) injection in the same animal. There is striatal TH terminal loss in the dorsal striatum on the side of ⟨-synuclein injection that appears more prominent for both S129 mutants (**E, H**) than for the wt (**B**). The distribution of α-synuclein-positive (LB509 antibody) striatal terminals (**C, F, I**) is similar among wild type (wt) and mutant recipient animals. Insets reveal high-power detail of ⟨-synuclein-positive nigrostriatal terminals. Bars indicate respective magnification ( ∫m).

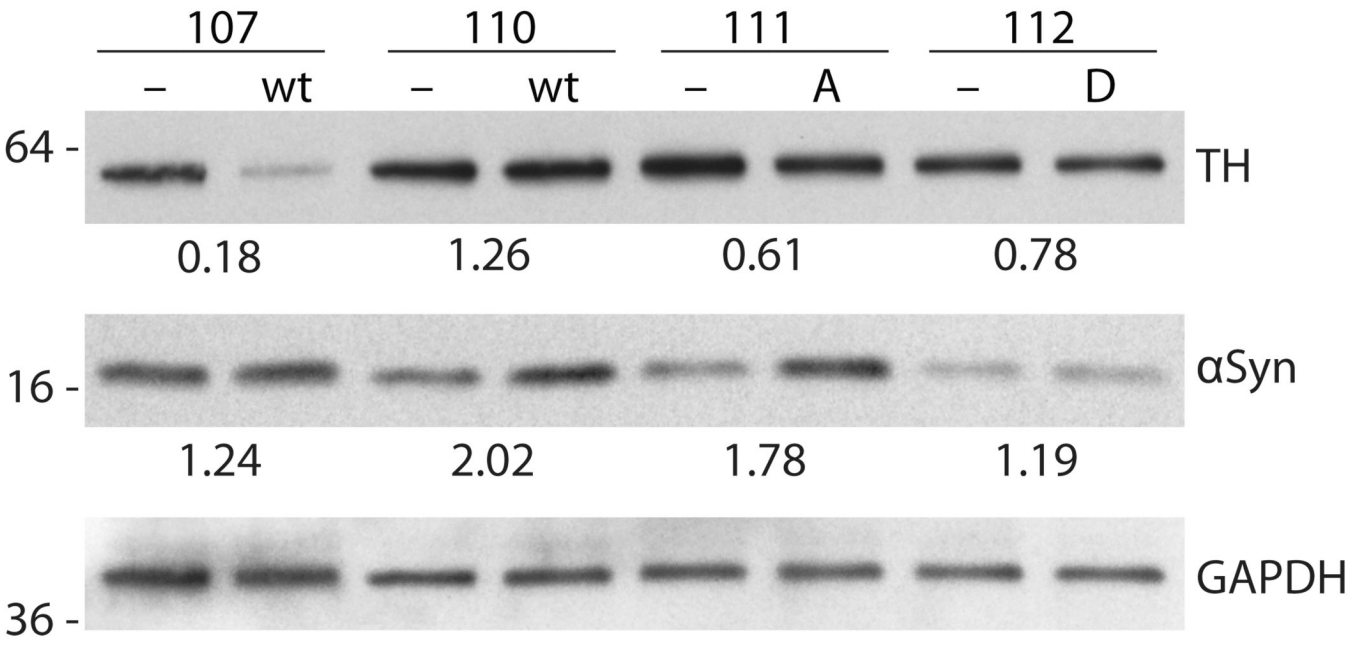



**Figure 3.**

Measurement of ⟨-synuclein and tyrosine hydroxylase (TH) expression in midbrain tissue 6 weeks post-viral injection. Western blots show samples from midbrain ipsilateral to ⟨-synuclein (wild type [wt], S129A [A], and S129D [D]) injection and are compared to protein extracts from the contralateral midbrain injected with empty vector (−) control in the same animal. Protein from left and right midbrain samples was probed with primary antibody to ⟨-synuclein and TH, followed by secondary HRP-conjugated antibody and ECL detection. Bands were quantified and normalized to GAPDH loading control. The ratio of ⟨-synuclein and TH for the α-synuclein (ipsilateral) versus empty vector (contralateral) side was calculated for each animal. Blots show ⟨-synuclein overexpression for most animals, but there was variable expression as shown for the 2 wt samples. Decreased TH expression is also shown for wt and S129 mutants.

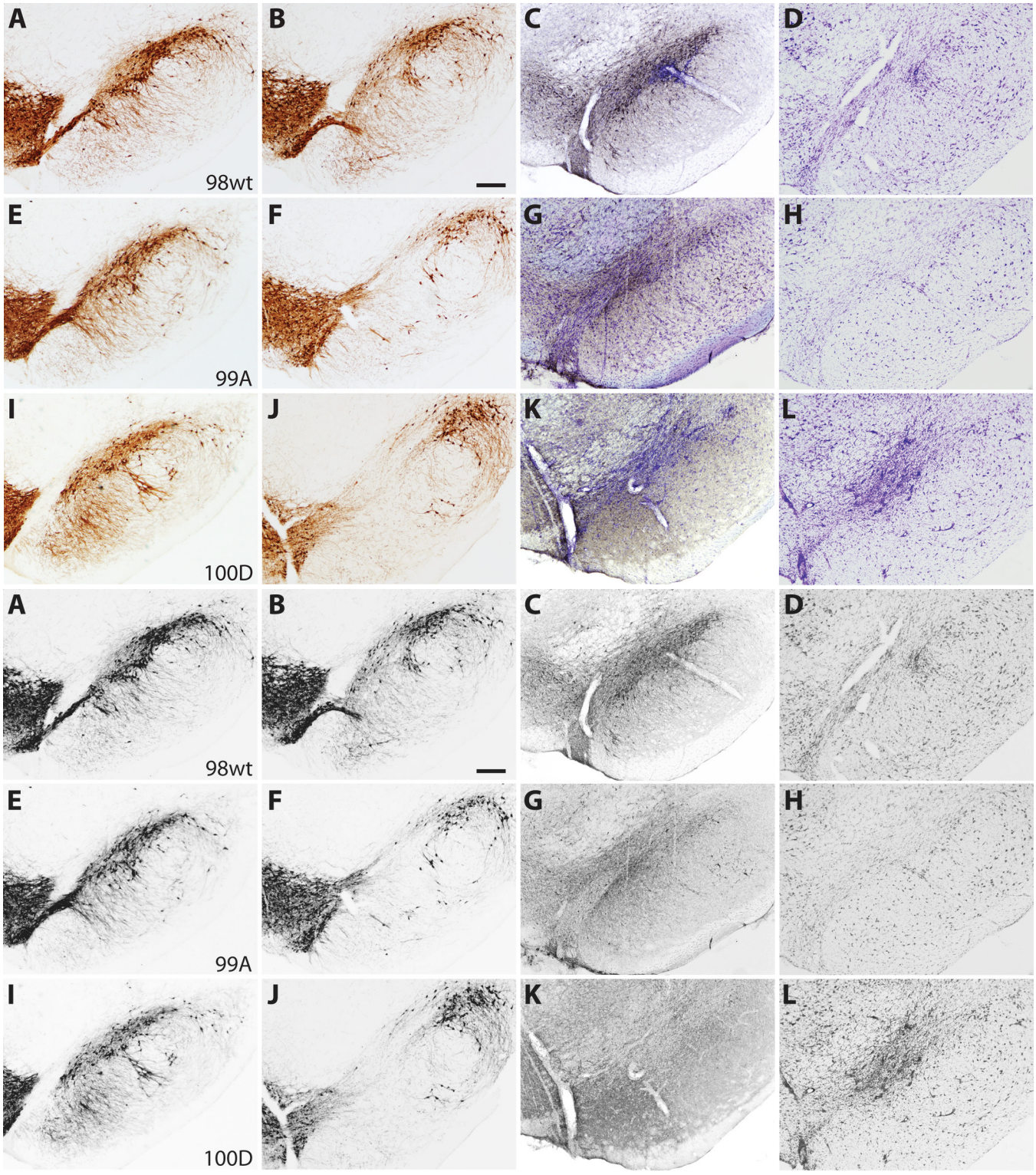



**Figure 4.**

Photomicrographs of representative substantia nigra (SN) sections at 6 weeks post-injection demonstrate neurodegenerative changes and tyrosine hydroxylase (TH) cell loss. (**A–D)** Case 98, wild type (wt) ⟨-synuclein. (**E–H)** Case 99, S129A. (**I–L)** Case 100, S129D. (**A, E, I)** TH

immunostaining in the contralateral SN after empty vector control injection. (**B, F, J)** TH cell loss in the SN for wt and S129 mutant cases. (**C, G, K**) Distribution of human ⟨-synuclein (LB509) expression (gray-black immunostaining) within the SN. (**D, H, L**) Adjacent sections stained with cresyl violet that demonstrate SN cell loss and gliosis at rAAV injection sites. Bar: 250 ⌠m.

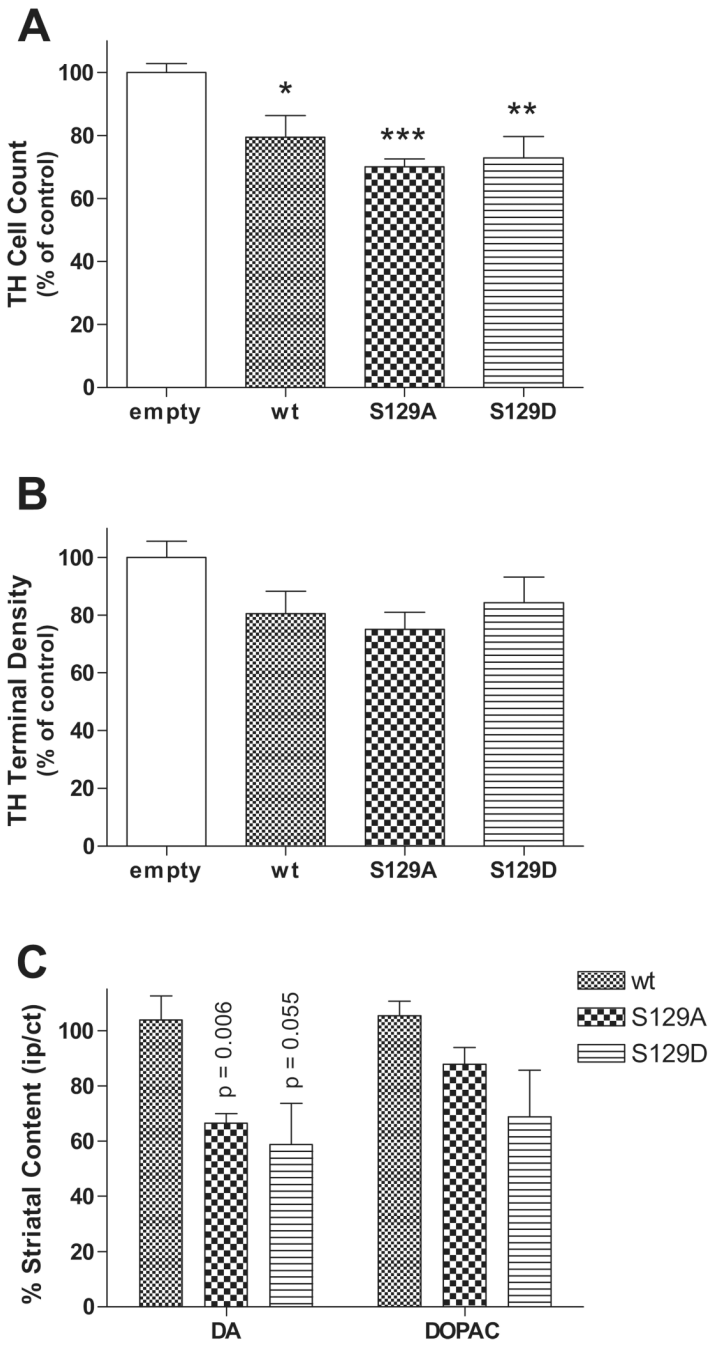



**Figure 5.**

Graphs of nigral tyrosine hydroxylase (TH) cell loss, striatal TH terminal density, and dopamine content. (**A**) Stereological estimates of TH cell loss in the SN at 6 weeks are expressed relative to empty vector control. Expression of both wild type (wt) and S129 mutant ⟨-synuclein resulted in significant TH cell loss (mean 25.8%) compared to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $F[3,24] = 9.96$, $p = 0.0002$). However, TH cells did not differ among wt and S129A and S129D groups. (**B**) Optical density scan array analysis of striatal TH terminal density showed a 16% to 25% decrease for wt and S129 mutants, but no significant difference among groups ($F[3,27] = 2.514$, $p = 0.080$). (**C**) Relative striatal dopamine (DA) (compared

to contralateral striatum injected with empty vector control) but not 3,4-dihydroxyphenlyacetic acid (DOPAC) content was reduced only in S129 mutants.

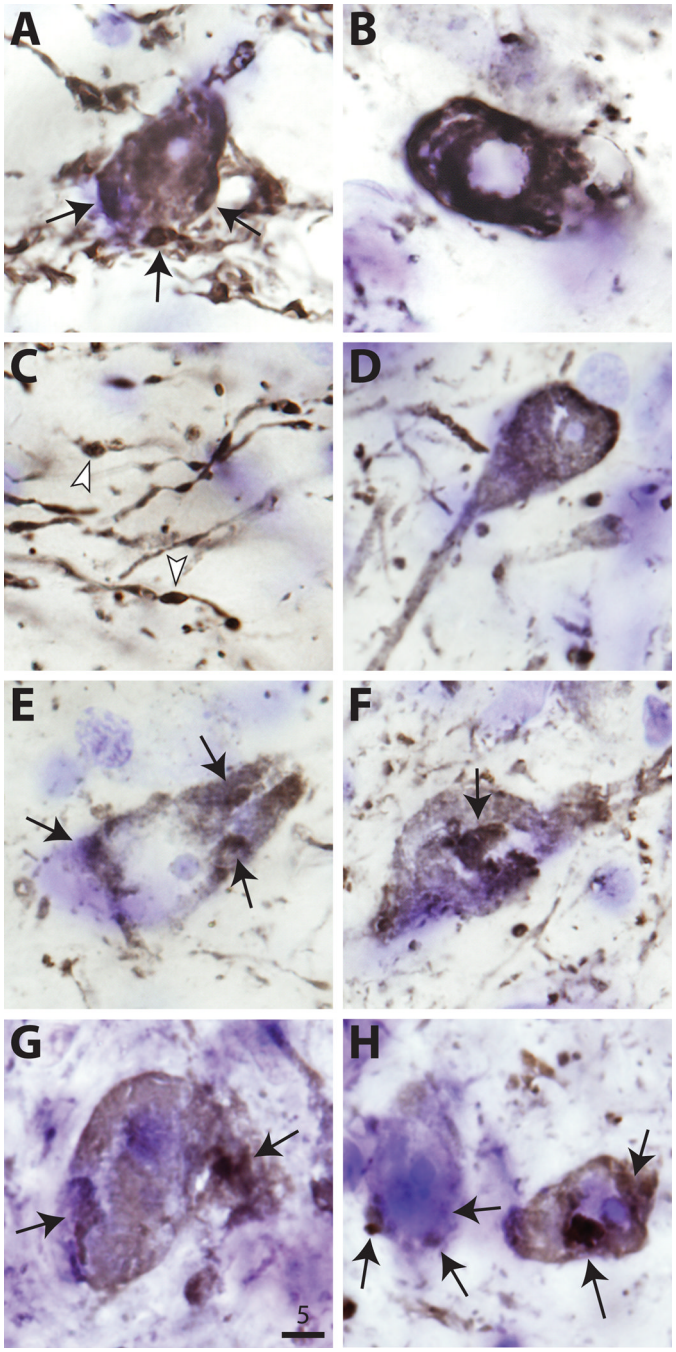



**Figure 6.**

High-power photomicrographs of human α-synuclein (LB509) immunostaining of nigral neurons in wild type (wt) (**A–C**), S129A (**D–F**), and S129D (**G, H**) recipients. Arrows indicate large intracytoplasmic ⟨-synuclein-positive aggregates. Most aggregates were either perinuclear or in the periphery (**A** and **G**). **B** shows typical nuclear ghosting, consistent with neurodegenerative change. Dystrophic neurites in **C** have characteristic α-synuclein-positive inclusions (arrowheads). Bar: 5 ⌠m.

Appendix T

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BRAIN RESEARCH

Research Report

# Deletion of adenosine $A_1$ or $A_{2A}$ receptors reduces L-3,4-dihydroxyphenylalanine-induced dyskinesia in a model of Parkinson's disease

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ABSTRACT

Adenosine $A_{2A}$ receptor antagonism provides a promising approach to developing nondopaminergic therapy for Parkinson's disease (PD). Clinical trials of $A_{2A}$ antagonists have targeted PD patients with L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) in an effort to improve parkinsonian symptoms. The role of adenosine in the development of LID is little known, especially regarding its actions via $A_1$ receptors. We aimed to examine the effects of genetic deletion and pharmacological blockade of $A_1$ and/or $A_{2A}$ receptors on the development of LID, on the induction of molecular markers of LID including striatal preprodynorphin and preproenkephalin (PPE), and on the integrity of dopaminergic nigrostriatal neurons in hemiparkinsonian mice. Following a unilateral 6-hydroxydopamine lesion $A_1$, $A_{2A}$ and double $A_1$–$A_{2A}$ knockout (KO) and wild-type littermate mice, and mice pretreated with caffeine (an antagonist of both $A_1$ and $A_{2A}$ receptors) or saline were treated daily for 18–21 days with a low dose of L-DOPA. Total abnormal involuntary movements (AIMs, a measure of LID) were significantly attenuated ($p<0.05$) in $A_1$ and $A_{2A}$ KOs, but not in $A_1$–$A_{2A}$ KOs and caffeine-pretreated mice. An elevation of PPE mRNA ipsilateral to the lesion in WT mice was reduced in all KO mice. In addition, neuronal integrity assessed by striatal dopamine content was similar in all KOs and caffeine-pretreated mice following 6-hydroxydopamine lesioning. Our findings raise the possibility that $A_1$ or $A_{2A}$ receptors blockade might also confer a disease-modifying benefit of reduced *risk* of disabling LID, whereas the effect of their combined inactivation is less clear.



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*Abbreviations:* AIM, abnormal involuntary movement; KO, knockout; L-DOPA, L-3,4-dihydroxyphenylalanine; 6-OHDA, 6-hydroxydopamine; LID, L-DOPA-induced dyskinesia; PD, Parkinson's disease; PPD, preprodynorphin; PPE, preproenkephalin; WT, wild-type.

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## 1. Introduction

Blockade of adenosine $A_{2A}$ receptors is being pursued as a non-dopaminergic alternative or adjunctive treatment of Parkinson's disease (PD). Several studies have investigated the usefulness of $A_{2A}$ receptor antagonism to treat L-DOPA-induced dyskinesia (LID), a complication from current PD therapy, in both animal models and clinical trials (Chen, 2003; Morelli et al., 2007). The use of $A_{2A}$ antagonists for symptomatic benefit with reduced risk of adverse effects in PD and LID is based *inter alia* on the discrete CNS distribution of the $A_{2A}$ receptor and its colocalization with $D_2$ receptors in the "indirect" pathway of the basal ganglia motor circuitry (Ferre et al., 1997; Kase, 2001; Fredholm et al., 2003). Elimination or blockade of $A_{2A}$ receptors expressed by forebrain neurons attenuates LID and related behaviors in hemiparkinsonian rodents or parkinsonian non-human primates (Bibbiani et al., 2003; Xiao et al., 2006).

Adenosine also activates the adenosine $A_1$ receptor, which–in contrast to discretely expressed $A_{2A}$ receptor–is widely distributed throughout the CNS including the hippocampus and cortex as well as on the striatal neurons of the "direct" and "indirect" pathways of the basal ganglia (Fastbom et al., 1987; Ferre et al., 1996; Johansson et al., 1997; Tohyama and Takatsuji, 1998) making a selective action difficult to deduce. It has been proposed that blocking $A_1$ receptors on striatonigral neurons of the direct pathway may facilitate motor activity by disinhibiting the motor stimulant actions of colocalized dopamine $D_1$ receptors on these neurons, whereas blocking $A_{2A}$ receptors on striatopallidal neurons of the indirect pathway may produce a parallel behavioral activation by mimicking the motor stimulant actions of colocalized $D_2$ receptors on these neurons (Ferre et al., 1997). By contrast, presynaptic $A_1$ and $A_{2A}$ receptors (e.g., those colocalized on corticostriatal nerve terminals) can inhibit and activate, respectively, adenylyl cyclase and thus transmitter release (van Calker et al., 1979; Olah and Stiles, 1995; Dunwiddie and Masino, 2001; Fredholm et al., 2005; Ciruela et al., 2006).

Thus adenosine may modulate LID through cooperative or opposing actions on two of its receptors in the CNS. To explore the roles of these receptors alone and in combination in a mouse model of LID in PD, we characterized single $A_1$ and $A_{2A}$ knockout (KO), as well as double $A_1$–$A_{2A}$ receptor KO phenotypes in (6-hydroxydopamine-lesioned) hemiparkinsonian mice treated daily with L-DOPA for three weeks. To avoid genetic background confounds mice were generated from double heterozygote crosses in a congenic C57Bl/6 background. In addition to this genetic approach to addressing adenosine receptor involvement in LID, we investigated the effect of pharmacological blockade of adenosine receptors using the widely consumed non-specific adenosine antagonist caffeine. The investigation of caffeine was prompted by preliminary clinical data that raised the possibility of a link between higher levels of caffeine consumption among PD patients and a reduced risk of subsequent dyskinesia development (Schwarzschild et al., 2003). We chose both a dose of caffeine, which elicits hyperlocomotion (15 mg/kg), and a lower dose of caffeine (3 mg/kg), which is capable of modifying neuroplasticity (as in that of conditioning preference) without necessarily producing a motor stimulant effect (Fredholm et al., 1999).

## 2. Results

### 2.1. *Effect of adenosine receptor knockout on 6-OHDA-induced neurotoxicity*

Previous studies showed that inactivation of $A_{2A}$ receptors by either genetic depletion or pharmacological blockade (caffeine and more specific $A_{2A}$ antagonists, but not a specific $A_1$ receptor antagonist) can protect against brain dopaminergic neurotoxicity induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Chen et al., 2001a,b). Accordingly, we first determined whether the dopaminergic lesion induced by 6-OHDA differs between control mice and KO or caffeine pre-treated mice. We found that genotype (Table 1) or pharmacological (Table 2) blockade did not affect levels of dopamine and its metabolite DOPAC in the 6-OHDA-lesioned and contralateral (non-lesioned) striata.

### 2.2. *Effect of adenosine receptor knockout on behavioral sensitization*

Following daily L-DOPA treatment, the hemiparkinsonian mice developed behavioral sensitization, as recorded by contralateral rotations and dyskinesia, quantified by an abnormal involuntary movements (AIMs) scale (Fig. 1). Acutely (on day 1), responses to L-DOPA were indistinguishable between adenosine receptor genotypes. Chronically, rotational sensitization on the plateau phase (days 11–21) in $A_1$ KO, $A_{2A}$ KO or $A_1$–$A_{2A}$ double KO mice showed a trend of attenuation over the 21 days, but the difference was not statistically significant ($p=0.12$, repeated measures model) in comparison

**Table 1 – Neurochemical measure of nigrostriatal innervation in wild-type and adenosine receptor KO mice chronically treated with L-DOPA after a unilateral 6-OHDA lesion.**

| Genotype | DA (pm/mg tissue) | DOPAC (pm/mg tissue) |
|---|---|---|
| WT ($n=12$) | | |
| Ipsilateral (lesioned) | 4.2±1.4* | 0.0±0.0* |
| Contralateral (intact) | 143.6±7.5 | 11.3±1.7 |
| $A_{2A}$ KO ($n=9$) | | |
| Ipsilateral (lesioned) | 4.3±3.0* | 0.7±0.5* |
| Contralateral (intact) | 129±10 | 15.9±7.4 |
| $A_1$ KO ($n=8$) | | |
| Ipsilateral (lesioned) | 6.9±3.0* | 0.4±0.4* |
| Contralateral (intact) | 120±14 | 9.7±0.9 |
| $A_1$–$A_{2A}$ KO ($n=7$) | | |
| Ipsilateral (lesioned) | 10.4±8.2* | 1.2±0.5* |
| Contralateral (intact) | 118±18 | 9.6±0.9 |

*$p<0.05$ versus respective contralateral (intact) striatum. The dopamine levels in the intact (right) side were not significantly different between wild-type and each of the three KO genotypes ($p>0.05$, Student's *t*-test).



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**Table 2 – Neurochemical measure of nigrostriatal innervation in unilateral 6-OHDA-lesioned mice treated daily with L-DOPA following pretreatment with saline or caffeine (3 mg/kg).**

| Treatment | DA (pm/mg tissue) | DOPAC (pm/mg tissue) |
|---|---|---|
| Saline ($n=21$) | | |
| Ipsilateral (lesioned) | 1.3±0.6* | 1.2±0.4* |
| Contralateral (intact) | 82.5±8.3 | 13.1±6.5 |
| Caffeine ($n=24$) | | |
| Ipsilateral (lesioned) | 3.6±1.7* | 0.3±1.3* |
| Contralateral (intact) | 88.8±10.6 | 14.8±7.6 |

*$p<0.05$ versus respective contralateral striatum. The dopamine levels in the intact (right) side were not significantly different between wild-type and each of the three KO genotypes ($p>0.05$, Student's $t$-test).

to WT (Fig. 1A). Total AIMs in $A_1$ KO, or $A_{2A}$ KO were attenuated significantly in comparison to WT ($p=0.0003$ for $A_1$ KO and $p=0.014$ for $A_{2A}$ KO, pairwise comparison with Bonferroni correction), but not in $A_1$–$A_{2A}$ double KO mice ($p=0.091$, pairwise comparison without correction) (Fig. 1B). The attenuation appeared incomplete in all KO genotypes, with responses in KO mice during the plateau phase still significantly increased compared to their initial (day 1) response ($p<0.05$, Fig. 1). Importantly, the effects of $A_1$ and $A_{2A}$ deletion on total AIMs were non-additive. In fact, for total AIMs, the $A_1$–$A_{2A}$ double KO mice appeared to have higher levels than either the $A_1$ KO or $A_{2A}$ KO mice at time points of days 11 and 14.

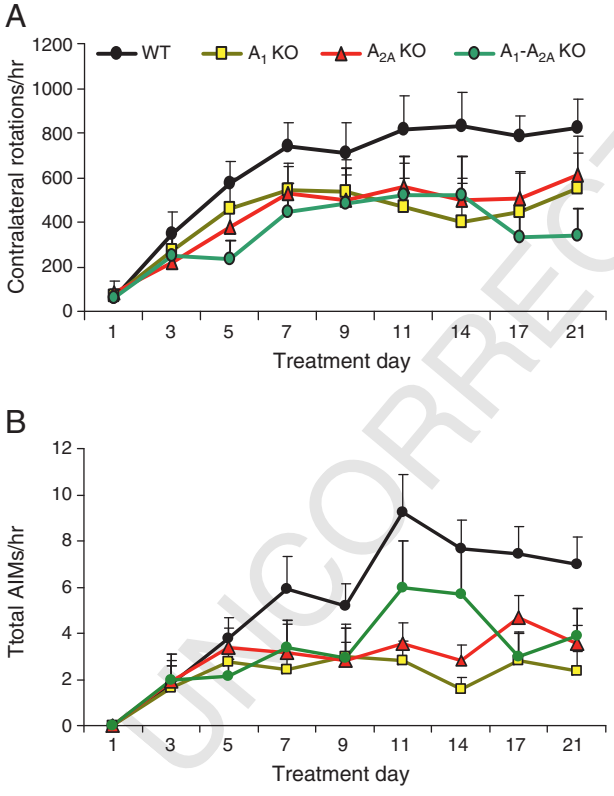


**Fig. 1 – Contralateral rotations and abnormal involuntary movements (AIMs) in $A_1$, $A_{2A}$ and $A_1$–$A_{2A}$ double KO mice compared to WT. Mice were treated daily with L-DOPA (2 mg/kg, i.p.) in combination with benserazide (2 mg/kg, i.p.) for 3 weeks. A, Contralateral turns in mice treated with L-DOPA. B, Total AIMs in mice treated with L-DOPA. Significant differences in the total AIMs in the plateau phase were observed between the WT ($n=12$) and the $A_1$KO ($n=9$), or $A_{2A}$KO ($n=8$) groups ($p<0.05$ for each comparison, Bonferroni correction).**

### 2.3. *Effect of caffeine on L-DOPA-induced behavioral sensitization*

Given the possible link between caffeine consumption among PD patients and a reduced risk of dyskinesia development (Schwarzschild et al., 2003), we tested the effect of caffeine on LID. In an initial experiment a low dose of caffeine (3 mg/kg, i.p.) significantly attenuated total AIMs (Fig. 2). By contrast, a higher caffeine dose (15 mg/kg, i.p.) did not significantly reduce the extent of AIMs that developed, though its chronic effects may have been confounded by its acute (day 1) stimulation of AIMs (as well as contralateral rotations) (Fig. 2A–B) The attenuation observed with low dose caffeine warranted efforts at replication and comparison to saline pretreatment. Despite the initial observation the pooled results of all three experiments with low dose of caffeine (Fig. 2C–D) showed no significant effect on AIMs that developed in response to repeated L-DOPA administration ($p>0.05$, repeated measures model), and similarly no effect on sensitized rotational responses ($p>0.05$, repeated measures model, Fig. 2C–D). The basis for this variability is not clear. However, an initial motor stimulant or potentiating effect, which was particularly prominent at the higher dose of 15 mg/kg caffeine, may confound any attenuating effect of caffeine on chronic contralateral rotations. On day 1, contralateral rotations in the caffeine group (17±6.0 per hour) were in fact significantly higher than that in saline group (0.4±0.2 per hour, Student's $t$-test, $p=0.017$; Fig. 2A). Not surprisingly and consistent with our previous finding (Yu et al., 2006), the higher dose of caffeine (15 mg/kg) produced an even greater contralateral rotation on day 1 (221±70 per hour) versus control (0.4±0.3 per hour; $p=0.019$), and did not significantly attenuate either L-DOPA-induced rotations or AIMs ($p>0.05$, Fig. 2).

### 2.4. *Modulation of striatal gene expression*

In WT mice striatal preproenkephalin (PPE) mRNA levels were significantly elevated on the 6-OHDA-lesioned side and showed a similar increase after lesioning with subsequent chronic L-DOPA treatment (Fig. 3), consistent with prior observations of the activation of PPE-expressing striatopallidal neurons with the development of LID in parkinsonian rodents and primates (Bedard et al., 1999; Zeng et al., 2000; Winkler et al., 2002; Morissette et al., 2006). Elevation of PPE following 6-OHDA lesioning in otherwise untreated WT mice was undiminished in $A_{2A}$ KO, but absent in all adenosine receptor KOs after chronic L-DOPA treatment. They showed significantly lower striatal PPE mRNA ipsilateral to 6-OHDA lesioning after chronic L-DOPA treatment compared to their WT littermates (Fig. 3). The PPD on the lesioned side tended to be reduced in



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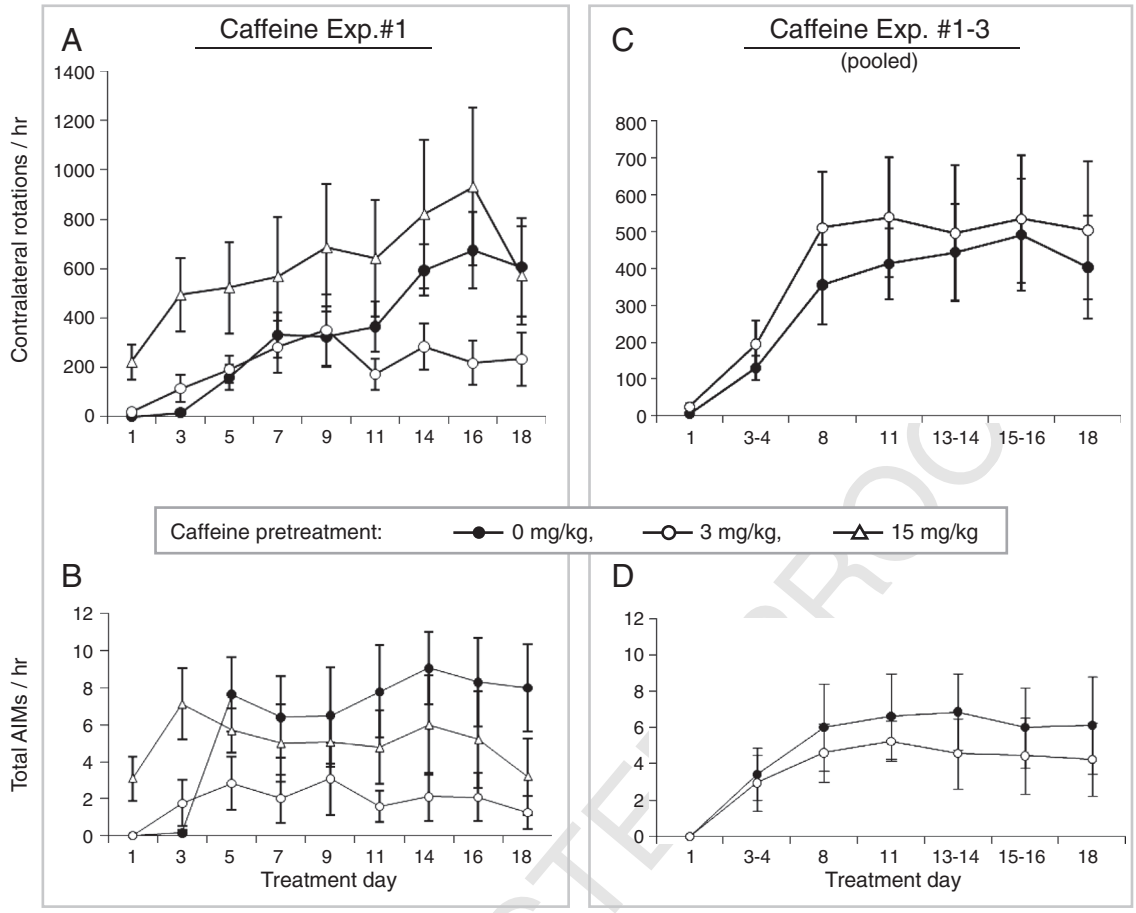


**Fig. 2 – L-DOPA-induced contralateral rotations and AIMs in caffeine-treated mice compared to saline controls. Mice were treated daily for 18 days with L-DOPA (2 mg/kg, i.p. in combination with benserazide 2 mg/kg, i.p.) 10 min after i.p. administration of saline or caffeine. (A, B) Rotational responses and total AIMs in an initial experiment with three treatment groups, 0 ($n=7$), 3 ($n=6$) or 15 ($n=7$) mg/kg caffeine ($p<0.05$, comparing 3 mg/kg caffeine to saline). (C, D) A composite of rotational responses and total AIMs from the initial and two subsequent experiments that compared only the low dose 3 mg/kg caffeine ($n=24$) with saline ($n=21$) pretreatments ($p>0.05$, comparing saline to caffeine).**

comparison to the non-lesioned side (Student's *t*-test, $p=0.07$) in $A_{2A}$ KO mice, consistent with our previous finding (Fredduzzi et al., 2002), in $A_1$ KO ($p=0.09$) and $A_1$–$A_{2A}$ KO ($p=0.09$) (data not shown). Comparison of these markers in the contralateral (non-lesioned) striatum showed no difference in PPE and PPD ($p>0.05$ for all KOs mice, Student's *t*-test) in comparison with WT control, suggesting that L-DOPA treatment had no differential effect on gene expression in unlesioned striata across genotypes.

To address the possibility of a change in prepeptides gene expression due to the gene KO itself, we also studied the baseline changes (without L-DOPA treatment or 6-OHDA lesioning), if any, of PPE and PPD in the colony of $A_1$, $A_{2A}$ and $A_1$–$A_{2A}$ double KO mice. We found that there was no difference of the striatal levels of peptide gene expression among the different genotypes ($p>0.05$, one-way ANOVA and Student's *t*-test, Fig. 4), consistent with previous findings (Chen et al., 1999).

Consistent with adenosine $A_1$–$A_{2A}$ receptor KO phenotype, low dose of caffeine (3 mg/kg) pretreatment tended to reduce the expression of PPE on the 6-OHDA lesioned side ($n=6$, $p=0.08$, Student's *t*-test), corresponding to caffeine's non-significant apparent effect on L-DOPA-induced dyskinesia. However, a higher dose of caffeine (15 mg/kg) had no effect on LID or PPE expression in comparison to the saline treated group ($p=0.71$, Student's *t*-test, data not shown).

## 3. Discussion

Genetic elimination of either adenosine receptor–either $A_1$ or $A_{2A}$–attenuated the development of AIMs in response to L-DOPA in a 6-OHDA lesion model of PD. However, a double KO of both adenosine receptor subtypes and caffeine had no clear effect on rotational sensitization and AIMs. In this 6-OHDA lesion model of PD and LID, striatal dopamine levels were unaffected in KO or caffeine-treated mice, suggesting that $A_1$ and $A_{2A}$ receptors facilitate its maladaptive neuroplasticity rather than the nigrostriatal lesion that is required for its full manifestation. In addition, induction of PPE, the activation of which in striatopallidal neurons links to the development of



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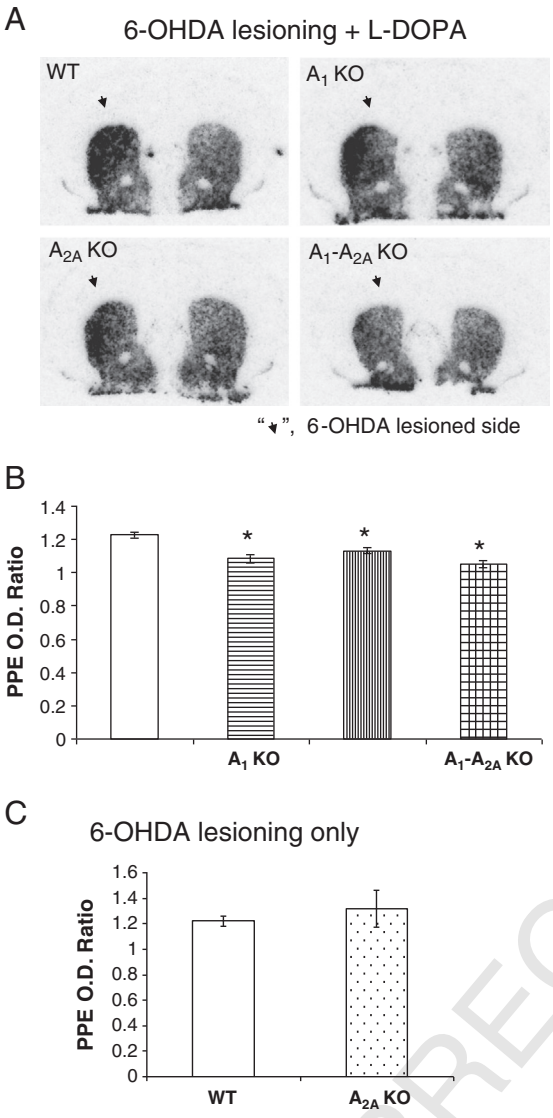


**Fig. 3 – Reduction of PPE mRNA in lesioned striata of adenosine receptor KO mice following chronic L-DOPA treatment. mRNA levels in the 6-OHDA-lesioned striata (arrow) were quantified as optical density (OD) at the mid-striatum level and expressed as a ratio to OD of the contralateral (unlesioned) striatum. (A,B) Chronic treatment with L-DOPA significantly reduced striatal PPE levels in 6-OHDA lesioned $A_1$ KO ($n=4$), $A_{2A}$ KO ($n=5$) and $A_1$-$A_{2A}$ KO ($n=4$) mice (*$p<0.05$; Student's $t$-test compared with the WT group, $n=5$). (C) 6-OHDA lesioning itself increased striatal PPE on the lesioned side in WT ($n=6$). This increase was not altered in $A_{2A}$ KO ($n=6$).**

LID in parkinsonian rodents and primates (Bedard et al., 1999; Zeng et al., 2000; Winkler et al., 2002; Morissette et al., 2006), was attenuated in adenosine receptor KO striata.

### 3.1. *The role of $A_1$ and $A_{2A}$ receptors in L-DOPA-induced dyskinesia*

Earlier studies have shown that blockade of $A_{2A}$ receptors can improve symptoms of PD and decrease neurodegenerative changes in PD models (Chen, 2003; Chen et al., 2007). Here we show that LID can be reduced by $A_{2A}$ receptor depletion, which produced no apparent protective phenotype in a 6-OHDA model in keeping with our previous findings (Fredduzzi et al., 2002; Xiao et al., 2006). $A_{2A}$ depletion has consistently shown no effect on the neurochemical (dopamine content) or neuroanatomical (dopamine transporter ligand binding) indicators of dopaminergic neuron injury in the unilateral 6-OHDA-leison model used here [(Fredduzzi et al., 2002; Xiao et al., 2006); Tables 1 and 2]. This lack of neuroprotection by $A_{2A}$ elimination in this relatively progressive model of PD contrasts the neuroprotective phenotype of the $A_{2A}$ receptor blockade seen with the more acutely acting neurotoxin MPTP (Chen et al., 2001a,b; Ikeda et al., 2002; Pierri et al., 2005; Carta et al., 2009) , and may reflect a greater opportunity for KO adaptation in the setting of a more gradual injury. In any event, the lack of neuroprotection against 6-OHDA in the $A_{2A}$ KO reduces the likelihood of a confounding attenuation in the nigrostriatal lesion in these mice. Thus the reductions in LID reported here suggest a role for adenosine receptors in the long-term maladaptive neuroplasticity underlying LID rather than in cell death.

The facilitative role of $A_{2A}$ in LID neuroplasticity may include both pre- and post-synaptic mechanisms, interacting with dopaminergic neurotransmission at a network level, as previously discussed (Fredduzzi et al., 2002; Xiao et al., 2006). The effects of blocking $A_1$ receptor in LID are less well understood, as there is clear evidence for multiple sites of presynaptic, as well as postsynaptic expression and actions of $A_1$ receptors in the striatum. For example, presynaptic striatal $A_1$ receptors inhibiting glutamate (Ambrosio et al., 1996; Ciruela et al., 2006), dopamine or acetylcholine release in the striatum and elsewhere might contribute to this complexity (Fredholm and Dunwiddie, 1988; Jin et al., 1993; Borycz et al., 2007). In addition, it is not known whether the absence of $A_1$ receptors alters the dopamine receptor function that contributes to LID.

Finally, the activation of PPE-expressing striatopallidal neurons is linked with the development of LID in parkinsonian rodents and primates (Bedard et al., 1999; Zeng et al., 2000; Winkler et al., 2002; Morissette et al., 2006). Several studies have described that 6-OHDA lesion itself produces an increase in PPE as well (Henry et al., 1999; Carta et al., 2002; Tel et al., 2002; Ravenscroft et al., 2004); for a review see (Xu et al., 2005). The increased PPE expression was attenuated in adenosine $A_1$ or $A_{2A}$ KOs compared to WT mice after chronic L-DOPA treatment, not after 6-OHDA lesioning alone in $A_{2A}$ KOs, suggesting an interaction of dopaminergic and adenosinergic systems and possibly accounting for the attenuation of LID, as discussed previously (Xiao et al., 2006). The activation of PPD-expressing striatonigral neurons is also linked with the development of LID in parkinsonian rodents (Cenci et al., 1998; Winkler et al., 2002). Surprisingly, $A_{2A}$ or $A_1$ receptor deletion had indistinguishable effects on striatal PPE and PPD expression despite the widely divergent distributions of these receptors and their effects on motor activity.

### 3.2. *$A_1$ and $A_{2A}$ receptor interactions in L-DOPA-induced dyskinesia*

Although the attenuation of LID was partial in both the $A_1$ and $A_{2A}$ KO mice, there was no clear additivity or synergy of their



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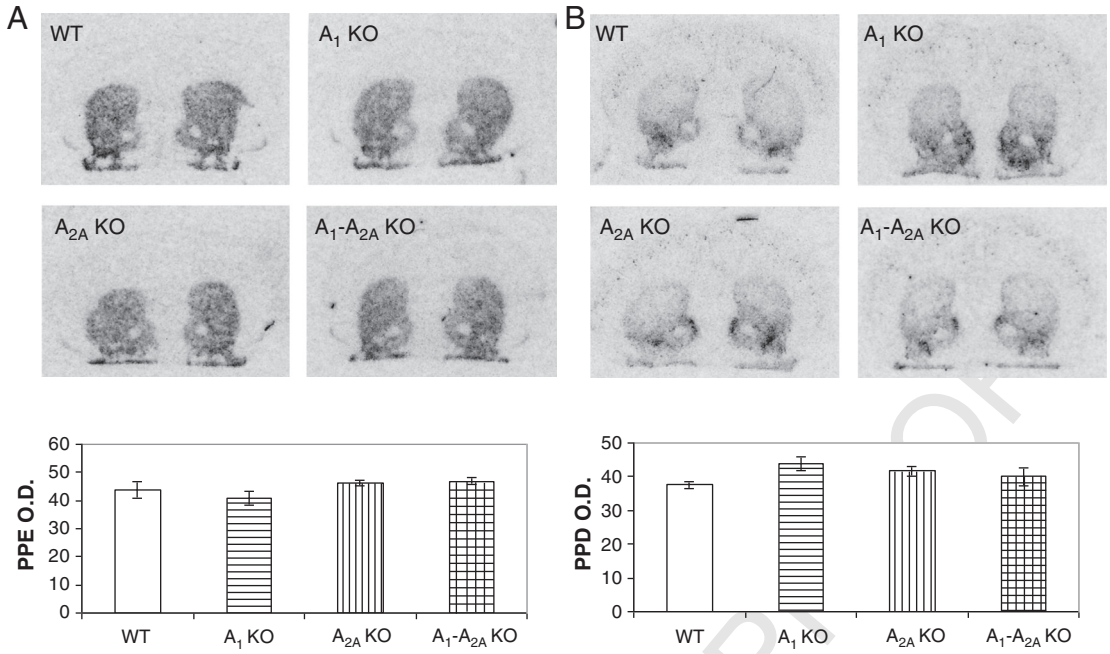


**Fig. 4 – Basal mRNA expression levels of PPE (A) and PPD (B) in WT, $A_1$ KO, $A_{2A}$ KO and $A_1$–$A_{2A}$ double KO mice. No differences in levels for either mRNA were observed among the different genotypes, WT ($n=6$), $A_1$ KO ($n=3$), $A_{2A}$ KO ($n=5$) and $A_1$-$A_{2A}$ KO ($n=4$) ($p>0.05$, one-way ANOVA).**

attenuating effects on LID in the double KO. In seeming contrast, the combination of $A_1$ and $A_{2A}$ antagonists (Karcz-Kubicha et al., 2003) produced an additivity of their individual motor-activating effects. Adenosine receptor mechanisms and hence their interactions in acute motor activation likely differ from those in LID. Similarly, less than additive effects of $D_1$ and $D_2$ dopamine receptor antagonists have been reported on complex adaptive behaviors, whereas acute effects of these drugs in combination are typically at least additive on motor activity (Schneider et al., 1991). In LID $A_1$ and $A_{2A}$ receptors may have sufficiently redundant effects in parallel to preclude an additional effect of blocking one receptor after the other has been inactivated. Alternatively, $A_1$ and $A_{2A}$ receptor roles in LID may occur sequentially—either when colocalized to a single cell or when interacting in different cells at a network level [(e.g. review by (Lopes et al., 2002; Xu et al., 2005)].

The results of this study suggest that both $A_1$ as well as $A_{2A}$ receptor antagonists could be useful therapeutically to lower the risk of L-DOPA-induced dyskinesia in PD patients. Our demonstration of a similar dependence on $A_1$ receptors prompted a complementary pharmacological test of the mixed $A_1$–$A_{2A}$ caffeine in this model of LID. We found that caffeine with the doses of 3 and 15 mg/kg showed no significant attenuation on L-DOPA-induced dyskinesia or rotational sensitization, possibly due to its general motor stimulant actions even at a low dose of 3 mg/kg considering significantly higher contralateral rotations on day 1 in mice pretreated with caffeine (Fig. 2). The initial motor stimulating effect may confound a chronic attenuation effect of caffeine on L-DOPA-induced behavioral sensitization. The results also suggest that blocking both $A_1$ and $A_{2A}$ receptors simultaneously, as occurs with caffeine use, may not provide a better prophylaxis against dyskinesia development than that of a specific $A_1$ or $A_{2A}$ antagonist alone.

## 4. Experimental procedures

### 4.1. *Generation of adenosine receptor KO mice*

Wild-type (WT) control ($A_1^{+/+}$, $A_{2A}^{+/+}$), $A_1$ KO ($A_1^{-/-}$, $A_{2A}^{+/+}$), $A_{2A}$ KO ($A_1^{+/+}$, $A_{2A}^{-/-}$) and $A_1$-$A_{2A}$ double KO ($A_1^{-/-}$, $A_{2A}^{-/-}$) mice were generated by double heterozygous mating ($A1^{+/-}$, $A_{2A}^{+/-}$ x $A_1^{+/-}$, $A_{2A}^{+/-}$) and genotyped by PCR analysis of tail DNA as described previously (Bastia et al., 2005; Kachroo et al., 2005; Xiao et al., 2006). The double heterozygotes were the offspring of crosses between homozygous $A_1$ KO and homozygous $A_{2A}$ KO mice from lines that we rendered congenic for the C57Bl/6 strain background (BanburyConference, 1997).

All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with an approval from the animal subjects review board of Massachusetts General Hospital. We have made all efforts to minimize the number of animals used and their suffering (along with giving the mice the best care).

### 4.2. *6-hydroxydopamine lesion of striatum in mice*

All mice were maintained in home cages with a 12 h light/dark cycle. The dopaminergic nigrostriatal pathway on the left side of each mouse was lesioned by a stereotactic intrastriatal infusion of 10 μg 6-hydroxydopamine (6-OHDA, hydrobromide, Sigma, St. Louis, MO) as described

previously (Fredduzzi et al., 2002; Xiao et al., 2006). On the day of surgery, mice were anesthetized with avertin-HCl (2% 2,2,2-tribromoethanol and 1% amyl alcohol; 10–15 ml/kg, i.p.). Two microliters of freshly prepared 6-OHDA bromide salt (5 μg/ μl in 0.05% ascorbic acid and shielded from light) were delivered by a microinfusion pump (0.5 μl/min) into the left striatum at the following coordinates (from bregma: 0.5 mm anterior, 2 mm lateral, and 2.8 mm ventral) (Franklin and Paxinos, 1997). To minimize damage to noradrenergic neurons, the mice were pretreated with desipramine hydrochloride (25 mg/kg, i.p.).

The mice were fed with both veterinary Nutri-Cal (Frenchtown, NJ) and human infant nutritional supplements (Enfamil) immediately after surgery for several days, followed by soft food (Bio-Serv Nutra-Gel Diet) to help ensure survival. Twelve days following surgery each mouse underwent a cylinder test (Cenci and Lundblad, 2007), in which asymmetry of forepaw placement on the inner wall of a plexiglass cylinder was used as an indirect assessment of lesion extent. Mice with <90% of dopamine loss (~15% of the lesioned mice) in the lesioned striatum (compared to the contralateral, non-lesioned striatum) were excluded from all data analysis (except for that of dopamine content).

The mice included in the final analyses of the KO experiment comprised 29 male and 9 female mice (3–14 months old with average age 7.5 months) and were balanced for age, weight and gender across genotypes ($n$=8–12 in each genotype group). Unlesioned WT and $A_{2A}$ KO mice were used for a control study of lesioning effects on neuropeptides, and comprised 7 male and 5 female mice (with an average age of 8 months) and were balanced for age, weight and gender across genotypes ($n$=6 in each genotype group). The mice included in the caffeine experiment comprised 52 males (3 months old, $n$=21 for saline, $n$=24 for low dose 3 mg/kg of caffeine, $n$=7 for high dose 15 mg/kg caffeine), and were balanced for home cage residence, cylinder test results and weight loss after lesion across treatment groups.

### 4.3. *Pharmacological treatment and behavioral testing*

Two weeks after 6-OHDA lesioning, mice were treated daily with L-DOPA (L-3,4-Dihydroxyphenylalanine, Sigma, St. Louis, MO, freshly prepared, 2 mg/kg i.p.) for 18–21 days. In the adenosine receptor KO experiment, benserazide (Sigma, St. Louis, MO, 2 mg/kg i.p. in saline), as previously used (Lundblad et al., 2004; Xiao et al., 2006), was administered 20 min prior to each dose of L-DOPA. In an initial caffeine experiment, 20 mice were divided into three groups: 0 ($n$=7), 3 ($n$=6) and 15 ($n$=7) mg/kg caffeine (Sigma, St. Louis, MO) i.p. in saline, which preceded by 10 min each daily i.p. dose of L-DOPA (2 mg/kg, mixed with benserazide 2 mg/kg in saline, to reduce the number of injections). Additional two caffeine experiments were performed at the lower 3 mg/kg dose with saline control ($n$=14) and caffeine ($n$=18, in saline) treatment groups. Pooled data from the three caffeine (low dose) experiments are presented (Fig. 2C–D).

Behaviors–total AIMs (including axial, limb and orolingual AIMs) (Cenci and Lundblad, 2005) and rotations–were recorded every 2–3 days for one hour . Dyskinetic behaviors were assessed and scored by an observer blind to the genotype or treatment, and based on each of the following subscale: 1, axial AIMs (i.e., twisted posture of the neck or the upper body toward the contralateral side); 2, forelimb AIMs (i.e. jerky movements or purposeless fluttering movement of the fore paw); 3, orolingual AIMs (i.e., twitching of orofacial muscles, empty jaw movements, and tongue protrusion), as previously established and validated (Lundblad et al., 2004). The AIM subscales were evaluated together 15, 30, 45 and 60 min after L-DOPA injection, and each mouse was observed for 1 min.

Both contralateral and ipsilateral turns were recorded immediately after L-DOPA administration for 60 min in 10 min time bins, using an automated rotometry system (San Diego Instruments, San Diego, CA), as described previously (Xiao et al., 2006). Briefly, each mouse was placed at the center of 1 of 12 opaque glass flat-bottom bowls and connected to the lower end of a cable tether by a rubber band snugly fitted around the chest. The upper end of the cable is attached to a swivel box linked to a computer interface.

### 4.4. *Molecular and neurochemical assessments*

One to three days after the last L-DOPA injection for both KO and caffeine experiments, whole brains were dissected out, frozen on dry ice and stored at –80 °C. The brains through the striata were sectioned with a cryostat (Shandon) at thickness of 12 μm as described before (Benn et al., 2004; Xiao et al., 2006). The rostral half of the striata was used for mRNA expression quantification of preproenkephalin (PPE) and preprodynorphin (PPD) by *in situ* hybridization histochemistry using radiolabeled oligonucleotide $^{35}$S, as described previously (Benn et al., 2004; Xiao et al., 2006). The probe sequence (5' to 3') for PPE mRNA was as follows: ATC TGC ATC CTT CTT CAT GAA ACC GCC ATA CCT CTT GGC AAT GAT CTC. The probe sequence (5' to 3') for PPD mRNA was as follows: ATG GGG GCT TCC TGC GGC GCA TTC GCC CCA AGC TGA AGT GGG ACA. Optical densities (O.D.) of the mRNA transcripts were quantified for the striatum using Image J 1.40 g software (National Institutes of Health, USA). Approximately three sections through the striatum, at rostral and middle levels, were analyzed for each mouse.

The remaining frozen brains containing the caudal half of the striata were sectioned to a thickness of 400 μm at –17 °C, and the striata were micropunched (Stoelting, 1.25 mm diameter). The cores from the left and right striata were analyzed by HPLC with electrochemical detection for dopamine (DA) and DOPAC content (Chen et al., 2001a,b; Xu et al., 2006).

### 4.5. *Statistical analysis*

All data are expressed as group average ± SEM. The difference between treatments (WT versus KO; caffeine versus saline) during the plateau phase of LID (from day 11 on for each analysis) was evaluated using a repeated measures model with a compound symmetry covariance structure. No significant time by treatment interaction



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was observed in any model, so only the main effect of group is reported. If a global difference between groups was observed, post-tests were performed with Bonferroni correction to account for multiple comparisons. A Wilcoxon signed rank test was used to compare each of the plateau measurements to the day 1 measurements for each genotype. The analysis was generated using SAS software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC) and GraphPad. Student's t-test was used for the remaining statistical analyses of molecular correlates and dopamine loss (Tables).

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CHAPTER 10

# Pathophysiological roles for purines: adenosine, caffeine and urate

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**Abstract:** The motor symptoms of Parkinson's disease (PD) are primarily due to the degeneration of the dopaminergic neurons in the nigrostriatal pathway. However, several other brain areas and neurotransmitters other than dopamine such as noradrenaline, 5-hydroxytryptamine and acetylcholine are affected in the disease. Moreover, adenosine because of the extensive interaction of its receptors with the dopaminergic system has been implicated in the pathophysiology of the disease. Based on the involvement of these non-dopaminergic neurotransmitters in PD and the sometimes severe adverse effects that limit the mainstay use of dopamine-based anti-parkinsonian treatments, recent assessments have called for a broadening of therapeutic options beyond the traditional dopaminergic drug arsenal.

In this review we describe the interactions between dopamine and adenosine receptors that underpin the pre-clinical and clinical rationale for pursuing adenosine $A_{2A}$ receptor antagonists as symptomatic and potentially neuroprotective treatment of PD. The review will pay particular attention to recent results regarding specific $A_{2A}$ receptor–receptor interactions and recent findings identifying urate, the end product of purine metabolism, as a novel prognostic biomarker and candidate neuroprotectant in PD.

**Keywords:** $A_{2A}$ receptors; neuroprotection; neurodegeneration; Parkinson's disease

## Adenosine receptors: localization and functional interactions with dopamine receptors

Adenosine acts in the brain via four cloned and pharmacologically characterized G-proteins' coupled receptors: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$, however, only the $A_1$ and $A_{2A}$ are thought to play an important role at physiological concentrations of adenosine in the CNS (Fredholm et al., 2005). The $A_1$ receptors has a widespread distribution in the brain whereas, of particular relevance for Parkinson's disease (PD), the $A_{2A}$ receptor is highly enriched in dopamine innervated areas.

High densities of adenosine $A_{2A}$ receptors are present in both the ventral and the dorsal striatum of rodents and primates, including humans. These

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receptors co-localize in the striatum with the dopamine $D_2$ receptor in the dendritic spines of enkephalin-rich striatopallidal gamma-aminobutyric acid (GABA) neurons and on glutamatergic terminals (Rosin et al., 1998; Schiffmann et al., 1991). This anatomical framework provides an important structural basis to our understanding of previously discovered $A_{2A}/D_2$ functional interactions.

In addition, $A_{2A}$ receptors are highly expressed in the globus pallidus (GP), mainly in the neuropil, where their stimulation enhances striatopallidal GABA outflow, and their blockade reduces it (Ochi et al., 2000; Rosin et al., 1998; Shindou et al., 2003). In 6-hydroxydopamine (6-OHDA)-lesioned rats, intra-pallidal infusion of $A_{2A}$ receptor antagonists, while not eliciting any motor response *per se*, does potentiate motor activity induced by L-DOPA (L-3,4-dihydroxyphenylalanine) or dopaminergic agonists. This suggests that blockade of pallidal $A_{2A}$ receptors, by reducing extracellular GABA, may stabilize GP activity and in turn subthalamic nucleus (STN) activity (Simola et al., 2006). Therefore, both structures may contribute to the therapeutic action of $A_{2A}$ receptor antagonists.

Adenosine $A_{2A}$ receptors exert an excitatory influence on striatopallidal neurons, in part through their antagonistic effect on dopamine $D_2$ receptor activation (Fig. 1). The basis of this antagonistic action of adenosine $A_{2A}$ receptors is their ability to decrease the binding affinity of $D_2$ receptors for dopamine as demonstrated in rat striatal membrane, in human striatal tissue and in different cell lines (Canals et al., 2003; Diaz-Cabiale et al., 2001; Ferré et al., 1991; Hillion et al., 2002). In agreement with these studies, stimulation of adenosine $A_{2A}$ receptors counteracts the $D_2$ receptor-mediated inhibition of cyclic adenosine monophosphate (cAMP) formation and $D_2$ receptor-induced intracellular $Ca^{2+}$ responses (Kull et al., 1999; Olah and Stiles, 2000; Salim et al., 2000). Of great importance, $A_{2A}$ receptors exert a strong influence on dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32), a DARPP, which is expressed at high levels in the GABAergic efferent neurons and is deeply involved in dopamine-mediated signalling (Lindskog et al., 2002) (Fig. 1).

The regulation of dopaminergic signal transduction by $A_{2A}$ receptors is also illustrated by the regulation of cAMP response element-binding (CREB) activity by $A_{2A}$ receptor stimulation, which increases cAMP formation and in turn phosphorylation of CREB. Selective $D_2$ receptor agonists dose dependently counteracted these effects (Kull et al., 1999). Furthermore, a variety of *in vivo* studies support the reciprocal antagonistic influence of $A_{2A}$ and $D_2$ receptors in induction of immediate early gene expression (e.g. *c-fos, zif/268*, NGFI-B and *jun-B*) (Morelli et al., 1995; Svenningsson et al., 1999; Tronci et al., 2006). Interestingly, the gene expression of striatal $A_{2A}$ receptors and the $A_{2A}/D_2$ receptor interaction are increased in the dopamine-denervated striatum (Ferré and Fuxe, 1992) (Fig. 1). Despite these mentioned findings, it has been shown that stimulation, as well as blockade, of adenosine $A_{2A}$ receptors induces behavioural and biochemical responses in mice lacking dopamine $D_2$ receptors, suggesting that adenosine $A_{2A}$ receptor actions can occur independently of dopamine signalling (Aoyama et al., 2000).

## Receptor–receptor interaction: heterodimeric complexes as basis for new anti-parkinsonian therapies

An important finding, with respect to $A_{2A}$ receptors, is the formation of functional receptor complexes (receptor mosaics) with other G protein-coupled receptors (GPCRs). $A_{2A}$ receptors, like many other GPCRs, form both homo and heterodimers (Agnati et al., 2003). This discovery has further increased our understanding of the biology of the $A_{2A}$ receptor with particular emphasis on molecular interactions with receptors for other neurotransmitters such as dopamine $D_2$, $D_3$, cannabinoid $CB_1$ and metabotropic glutamate mGlu5 receptors (Fig. 1). Heterodimerization may have functional and pharmacological consequences;

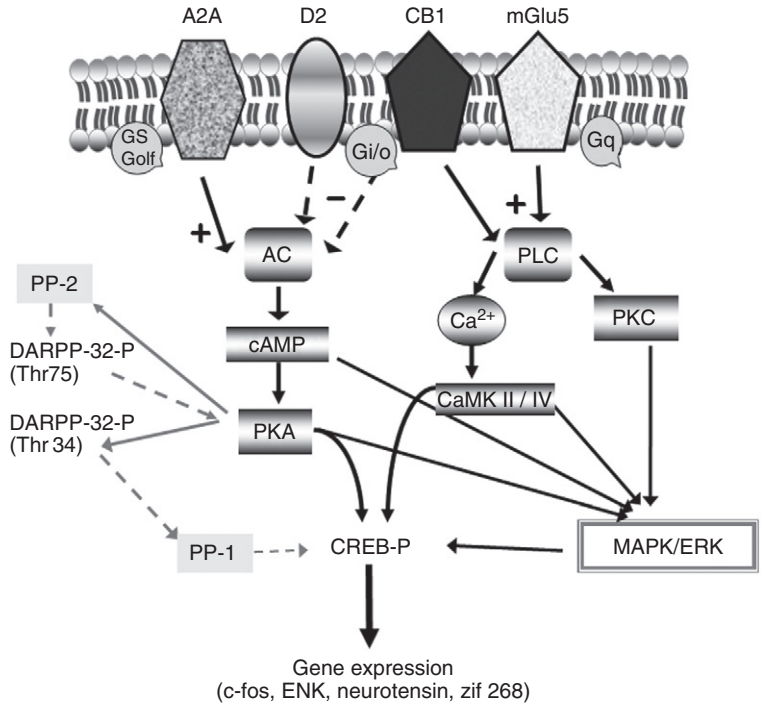


Fig. 1. Functional interactions between dopamine $D_2$, adenosine $A_{2A}$, cannabinoid $CB_1$ and glutamate mGlu5 receptors in striatopallidal neurons. Adenosine $A_{2A}$ receptors interact antagonistically with $D_2$ and $CB_1$ receptors at the intramembrane level and at the adenylyl cyclase level; metabotropic glutamate mGlu5 and adenosine $A_{2A}$ receptors act synergistically to counteract the $D_2$ dopamine receptor signalling in striatopallidal neurons. Synergistic interactions exist between $A_{2A}$ and mGlu5 receptors at the level of c-fos expression, MAP kinases and phosphorylation of DARPP-32 protein; for further explanation see text. broken arrows, inhibitory effect; '+', stimulation; '–', inhibition; AC, adenylyl cyclase; $Ca^{2+}$, calcium ions; CaMK II/IV, calcium/calmodulin-dependent protein kinase type II/IV; cAMP, cyclic AMP; CREB, cAMP response element-binding protein; $K^+$, potassium channel; DARPP-32, dopamine- and cAMP-regulated phosphoprotein; DARPP-32-P (Thr75) and DARPP-32-P (Thr34), DARPP-32-phopshorylated at threonine residues 75 and 34, respectively; Gi, Go, inhibitory G proteins; Gq, Gs, Golf, stimulatory G proteins; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP-1, protein phosphatase-1; PP-2, protein phosphatase-2.

therefore, the presence of heterodimeric complexes has constituted a considerable step forward in the neurobiology of adenosine, suggesting new ways of modulating neuronal activity by targeting the $A_{2A}$ receptor (Ferré et al., 2009; Fuxe et al., 2003).

Co-immunoprecipitation studies with fluorescence resonance energy transfer (FRET) and BRET (Bioluminescence Resonance Energy Transfer) analyses have demonstrated the existence of constitutive $A_{2A}/D_2$ heteromers as well as $A_{2A}$ homodimers within the plasma membrane, indicating less than 10 nm separating the receptors (Canals et al., 2003; Hillion et al., 2002).

Functional implications of the $A_{2A}/D_2$ intramembrane receptor–receptor interaction through heteromerization include a decrease in receptor affinity for dopamine agonists acting on $D_2$ receptors, as well as a reduction of $D_2$ receptor G protein coupling and signalling. Thus, the essence of this $A_{2A}/D_2$ receptor heteromerization may be to convert the $D_2$ receptor into a state of strongly reduced functional activity. The discovery of heteromeric

$A_{2A}/D_2$ complexes has added to the substantial evidence for antagonistic molecular, cellular, electrophysiological and behavioural interactions between $A_{2A}$ and $D_2$ receptors. These form the basis for anti-parkinsonian strategies that simultaneously block adenosine $A_{2A}$ and stimulate dopamine $D_2$ receptors (Morelli et al., 2007, 2009).

Moreover, evidence for functional $A_{2A}/D_3$ heteromers has recently been obtained in co-transfected $A_{2A}/D_3$ cells using FRET. $A_{2A}$ activation reduces the affinity of $D_3$ agonist binding sites as well as $D_3$ signalling (Torvinen et al., 2005). It should be noted, however, that while this interaction could provide interesting clues for mediation of ventral striatal (limbic) functions, it might not be relevant for dorsal striatum functions where the $D_3$ receptor, which is expressed only after dopamine denervation, and the $A_{2A}$ receptor are segregated in different neuronal populations (of striatonigral and striatopallidal neurons, respectively) (Bordet et al., 2000).

Although functional properties of multiple receptor heteromers remain to be determined, the interaction of $A_{2A}$ receptors with receptors other than those for dopamine is also relevant (Fig. 1). Co-immunoprecipitation evidence shows that $A_{2A}$ and mGlu5 receptors form heteromeric complexes, and combined stimulation of both of these receptor types synergistically reduced the affinity of the $D_2$ receptor agonist binding sites in striatal membranes (Fuxe et al., 2003). These observations were supported by the high degree of $A_{2A}$ and mGlu5 co-localization in primary cultures of striatal neurons and in striatal glutamatergic nerve terminals (Rodrigues et al., 2005). Co-activation of $A_{2A}$ and mGlu5 receptors causes a synergistic interaction at the level of c-*fos* expression and on extracellular signal-regulated kinases (ERK) as well DARPP-32 phosphorylation, indicating a possible role of this heteromeric complex in striatal plasticity (Ferré et al., 2002; Nishi et al., 2003) (Fig. 1). Combined $A_{2A}$ and mGlu5 receptor activation may also produce synergistic cellular effects on striatal output neurons *in vivo*, as demonstrated by a greater than additive increase in GABA release from ventral striatopallidal neurons after local perfusion with both $A_{2A}$ and mGlu5 agonists (Diaz-Cabiale et al., 2002). Similarly, the discovery of functional $A_{2A}$/mGlu5 receptor interactions and heteromeric $A_{2A}$/mGlu5 complexes led to recent findings of a synergistic anti-parkinsonian potential of combining $A_{2A}$ and mGlu5 antagonists (Coccurello et al., 2004; Kachroo et al., 2005).

Another interesting interaction that may have important implications for the design of new drugs useful in the treatment of PD is that of adenosine or dopamine receptors with cannabinoid $CB_1$ receptors whose presence has been described in basal ganglia, most specifically in GABAergic striatal neurons (Egertova and Elphick, 2000) (Fig. 1). $CB_1$ receptors co-localize with $D_2$ and $A_{2A}$ receptors predominantly in the soma and dendrites of the striatopallidal GABA neurons and in corticostriatal glutamate terminals. $CB_1$–$D_2$ heteromers as well as $CB_1$–$A_{2A}$ heteromeric complexes have been described in HEK-293 cell lines (Ferré et al., 2009; Marcellino et al., 2008). Interestingly post-synaptic $CB_1$ receptor signalling was found to be dependent on $A_{2A}$ receptor activation. Accordingly, blockade of $A_{2A}$ receptors counteracted the motor depressant effects and extracellular field potentials, in corticostriatal neurons, produced by cannabinoid $CB_1$ agonist (Carriba et al., 2007; Tebano et al., 2009). At the same time, CB1 receptors mediate psychomotor activation by $A_{2A}$ receptors antagonists (Lerner et al., 2010).

Antagonistic $CB_1/D_2$ interactions have been described at the behavioural level as well. The $CB_1$ receptor agonist CP 55940 at a dose that did not change basal locomotion is able to block quinpirole-induced increases in locomotor activity. In addition, not only the $CB_1$ receptor antagonist rimonabant but also the specific $A_{2A}$ antagonist MSX-3 blocked the inhibitory effect of $CB_1$ receptor agonists on $D_2$-like receptor agonist-induced hyperlocomotion (Marcellino et al., 2008). These results find support in the existence of antagonistic $CB_1/D_2$ receptor–receptor interactions within $CB_1/D_2$ heteromers in which $A_{2A}$ receptors

might also participate (Marcellino et al., 2008) (Fig. 1). Based on this biochemical evidence showing how $CB_1$ receptors interact with both $A_{2A}$ and $D_2$ receptors, it has been proposed that these receptors are putative targets for PD.

## Role of adenosine receptors in neuroprotection

Arresting disease progression at the level of its underlying neuronal degeneration remains a critical unmet goal of neurotherapeutics for PD. More than a decade of research has suggested that manipulating adenosine neurotransmission might offer a valuable strategy to achieve neuroprotection in PD.

The first studies investigating adenosine and neuroprotection were conducted in models of ischemic and excitotoxic brain injury [reviewed in Fredholm et al. (2005)]. Under these conditions, increased extracellular adenosine in response to brain injury has been shown to act as a neuroprotectant (Dux et al., 1990; Evans et al., 1987). However, a pro-neurotoxic role of adenosine has also been demonstrated, suggesting that blockade of adenosine receptors may confer neuroprotection across a range of neurodegenerative disorders (Chen et al., 1999; de Mendonca et al., 2000; Jones et al., 1998; Melani et al., 2003; Pinna et al., 2010; Popoli et al., 2002). This apparent paradox reflects the complexity of adenosine transmission, with several receptor sub-types selectively localized in brain areas and uniquely coupled to G proteins and signalling pathways, as described above. Moreover, the important role played by adenosine in the immune response in the central nervous system (CNS) should be considered as a component of degenerative and protective processes. Hence, the same adenosine receptor sub-type expressed in different cell types such as neurons and glia may mediate opposing effects in response to different neurotoxic insults (Fig. 2).

In PD, the initial suggestion that manipulating adenosine neurotransmission might be beneficial in terms of affecting disease onset or progression came from the epidemiological evidence that consumption of caffeine, a non-specific $A_1/A_{2A}$ receptor antagonist, is associated with a reduced risk of developing PD (Ascherio et al., 2001; Schwarzschild et al., 2003b). Convergent laboratory studies investigating the effect of caffeine and more specific adenosine receptor antagonists suggested that blockade of the $A_{2A}$ receptor sub-type prevents nigrostriatal degeneration in several models of PD (Schwarzschild et al., 2006). Interestingly, epidemiological studies have identified a second purine as another robust inverse risk factor for PD. Blood levels of urate (the end product of adenosine metabolism in humans) are also strongly linked to a reduced risk of PD and, more recently, of its progression, as described below.

### *Neuroprotection and $A_1$ adenosine receptors*

Preclinical evidence for a role of $A_1$ receptors in neuroprotection in PD is sparse; therefore this section will focus mostly on a general role of $A_1$ receptors on glutamate release and toxic cytokine control.

$A_1$ adenosine receptors are widely distributed throughout the CNS, being expressed both in neuronal and in glial cells (Daré et al., 2007; Ochiishi et al., 1999; Svenningsson et al., 1997). Neuronal $A_1$ adenosine receptors exist in pre-synaptic terminals as well as in post-synaptic membranes. However, the most striking effect of their stimulation is the inhibition of neurotransmitter release, mediated by a reduction of pre-synaptic calcium influx (Borycz et al., 2007; Brown et al., 1990; Masino et al., 2002; Ochiishi et al., 1999). Although similar effects have been reported for different neurotransmitters, including glutamate, dopamine, acetylcholine and GABA, the inhibition of glutamate release holds the main interest for neurodegeneration. Based on this mechanism, $A_1$ receptor agonists have been mainly pursued for their potential to protect under conditions characterized by a massive release of glutamate,

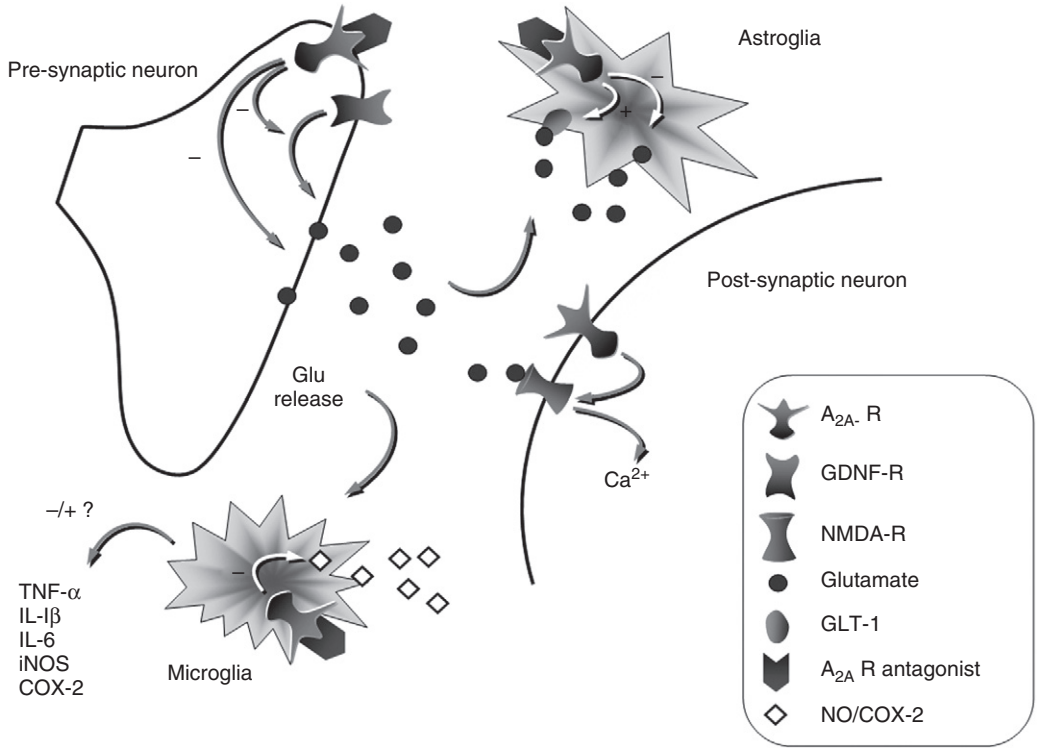


Fig. 2. Schematic representation of possible cellular mechanisms affected by $A_{2A}$ receptor blockade in a neuroprotective model of PD. In pre-synaptic neurons, $A_{2A}$ antagonism inhibits glutamate efflux either directly or indirectly through an inhibition of GDNF receptors (see text for more details on this mechanism). A decrease in glutamate release results in reduced glial response and as a consequence release of toxic factors. In microglia, direct $A_{2A}$ receptor blockade inhibits NO and COX-2 production. In astroglia, direct $A_{2A}$ receptor blockade inhibits glutamate release directly or indirectly through an inhibition of GLT-1.

as occurs in ischemic stroke. Few studies have investigated the effects of $A_1$ receptor agonism in models of PD. Lau and Mouradian (1993) have shown that an adenosine $A_1$ agonist was able to prevent the decrease in striatal dopamine levels induced by a single injection of the neurotoxin 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Accordingly, the $A_1$ receptor antagonist 8-cyclopentyl-1,3-dipropylxathine (CPX) may have slightly exacerbated striatal MPTP toxicity (Chen et al., 2001). Although the mechanism has not been fully clarified, an inhibition of excessive release of glutamate, which may contribute to MPTP toxicity, might be involved in the protective effect displayed by $A_1$ receptor agonists in the striatum. In line with this interpretation, blockade of N-methyl-D-aspartic acid (NMDA) glutamate receptors achieved a similar protective effect against an acute MPTP insult (Chan et al., 1993). Other clues to $A_1$-mediated neuroprotection in PD models came from evidence that caffeine pre-treatment protected against methamphetamine-induced nigrostriatal toxicity via an upregulation of $A_1$ receptors (Delle Donne and Sonsalla, 1994). Accordingly, Alfinito and colleagues have shown that administration of an $A_1$ antagonist exacerbated dopamine neuron degeneration induced by the mitochondrial inhibitor malonate, although nigral but not striatal $A_1$ receptors were selectively involved in the effect (Alfinito et al., 2003).

Glial $A_1$ receptors (Haskó et al., 2008) may play an important role in dopamine neuron demise in PD. The involvement of this receptor in neuroinflammatory responses has been well documented, although direct evidence towards its involvement in neuroinflammation associated with nigrostriatal damage is lacking. In the presence of ischemia and brain injury, extracellular adenosine stimulates glial $A_1$ receptors, resulting in inhibition of astrocyte proliferation and excessive reactive astrogliosis, as well as increased production of trophic factors, such as nerve growth factor (NGF), S100beta protein and transforming growth factor beta, which in turn may help to protect neurons from injury. Interestingly, the anti-inflammatory cytokine IL-6 enhances the expression of $A_1$ receptors in astrocytes, suggesting that a self-modulating loop involving $A_1$ receptors is triggered in presence of a neuronal damage (Ciccarelli et al., 2001; Schubert et al., 1997; van Calker and Biber, 2005). Although the topic is still poorly investigated, based on this evidence it is tempting to speculate that a modulation of the astroglial response through the $A_1$ receptor might be beneficial in PD nigrostriatal degeneration.

### *Neuroprotection and adenosine $A_{2A}$ receptors*

In contrast to the $A_1$ receptor, the $A_{2A}$ receptor has been consistently implicated as a mediator or modulator of dopaminergic neuron degeneration across a range of laboratory models of PD.

An early suggestion of the neuroprotective potential of $A_{2A}$ receptor blockade in PD came with the demonstration that caffeine can attenuate the loss of striatal dopamine induced by acute MPTP administration in mice (Chen et al., 2001). Caffeine has also been shown to be neuroprotective in other models of PD such as the unilaterally 6-OHDA-lesioned rat (Joghataie et al., 2004) and more recently in a dual pesticide, chronic exposure model (Kachroo et al., 2010), in which repeated systemic administration of paraquat plus maneb leads to degeneration of nigral dopaminergic neurons. In addition, two major demethylation metabolites of caffeine, namely theophylline and paraxanthine, both adenosine receptor antagonists themselves, have also been shown to protect against MPTP-induced neurotoxicity in mice (Xu et al., 2010). These consistent findings of neuroprotection by caffeine and its metabolites in multiple models of PD have strengthened the hypothesis that a true protective effect of caffeine is the basis for its inverse association with PD risk in epidemiological studies (see below).

A similar protective effect was observed upon administration of the selective $A_{2A}$ receptor antagonist KW-6002, but not with the $A_1$ receptor antagonist CPX (Chen et al., 2001; Pierri et al., 2005; Schwarzschild et al., 2006). In line with these results, genetic deletion of $A_{2A}$ receptors prevented the loss of striatal dopamine induced by acute MPTP (Chen et al., 2001). Thereafter, a protective effect by $A_{2A}$ antagonists was reported in a different toxin model of PD, in which 6-OHDA is infused within the rat striatum (Ikeda et al., 2002). In these studies, neuroprotection was assessed as attenuation of the striatal dopamine depletion or of the loss of tyrosine hydroxylase (TH)-positive cells in the substantia nigra *pars compacta* (Table 1). Recently, the $A_{2A}$ receptor antagonists SCH-58261 and ANR 94 were shown to prevent the death of nigral dopaminergic neurons induced by sub-chronic MPTP administration in mice (Carta et al., 2009; Pinna et al., 2010). Therefore, blockade of the $A_{2A}$ receptor seems to confer a functional protection of striatal dopamine transmission, as well as to prevent the loss of nigral dopaminergic neurons induced by neurotoxin exposure.

Despite considerable evidence suggesting the neuroprotective potential of $A_{2A}$ receptor blockade in PD, the underlying mechanism is still a matter of debate. In neurons, $A_{2A}$ adenosine receptors have been identified both pre- and post-synaptically, where they control neurotransmitter release and neuronal stimulation,

Table 1. Neuroprotective outcome of $A_{2A}R$ manipulation in different rodent PD models

| PD model | A2AR manipulation | Functional protection in Str | SNc neurons survival | Attenuation of glial response | References |
|---|---|---|---|---|---|
| 6-OHDA in Str | $A_{2A}$ antag. | + | + | NA | Ikeda et al. (2002) |
| MPTP acute | $A_{2A}$ antag. | + | + | + | Chen et al. (2001) |
| | tot$A_{2A}$KO | + | NA | NA | Ikeda et al. (2002) |
| | fbn$A_{2A}$KO | – | NA | NA | Pierri et al. (2005) Yu et al. (2008) |
| MPTP sub-chronic | $A_{2A}$ antag. | + | + | + | Carta et al. (2009) |
| | fbn$A_{2A}$KO | + | + | + | |

Parkinson's disease, PD; antag., antagonists; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine.
Summary of recent data highlighting the neuroprotective outcome observed in both the striatum and the substantia nigra by pharmacologically and genetically targeting the $A_{2A}$ receptor in different PD models.

respectively (Rebola et al., 2005; Rosin et al., 1998; Schiffman et al., 1991; Svenningsson et al., 1999) (Fig. 2). Moreover, cells involved in the neuroinflammatory response such as astroglia, microglia and bone marrow-derived cells all express the $A_{2A}$ receptor (Fiebich et al., 1996; Saura et al., 2005). Since many factors may contribute to neuronal demise in PD, including neuronal pathological processes and chronic neuroinflammation, several mechanisms have been investigated to explain the neuroprotective effect of $A_{2A}$ receptor blockade. Interestingly, recent reports have highlighted that multiple mechanisms, involving either neuronal or glial receptors, may be recruited in different PD models to achieve neuroprotection by $A_{2A}$ receptor blockade (Carta et al., 2009; Yu et al., 2008).

### *Neuronal $A_{2A}$ receptors and neuroprotection*

An alternative strategy employed to complement pharmacological approaches in demonstrating the neuroprotective efficacy of $A_{2A}$ receptor blockade has been offered by targeted gene mutations producing mice lacking this receptor. In general, $A_{2A}$ receptor knockout mice display attenuated brain damage in models of ischemia or excitotoxin-induced brain injury as had been previously observed using traditional pharmacological antagonists (Chen et al., 1999; Monopoli et al., 1998; Pedata et al., 2005; Phillis, 1995). More recently, studies using a conditional knockout (Cre/*loxP*) system to generate mice with a selective post-natal depletion of forebrain neuronal $A_{2A}$ receptors have contributed to the understanding of cellular mechanisms of $A_{2A}$ receptor-mediated neuroprotection in PD. In a sub-chronic MPTP model of PD, loss of these $A_{2A}$ receptors fully prevented neurotoxin-induced degeneration of nigral dopaminergic neurons, endorsing a primary role of neuronal $A_{2A}$ receptors in the neuroprotective effects of $A_{2A}$ antagonists in this model (Carta et al., 2009). However, neuronal $A_{2A}$ receptors seemed to play a minor role in striatal dopamine loss induced by a more acute MPTP intoxication, since Yu et al. (2008) reported that neuronal $A_{2A}$ receptors inactivation did not protect striatal terminals from a one-day, high-dose MPTP exposure (Yu et al., 2008).

Interestingly, in this study mice globally lacking $A_{2A}$ receptors were partially protected against striatal MPTP toxicity. This suggests that blockade solely of neuronal $A_{2A}$ receptors might not be sufficient to prevent neurotoxicity induced by acute MPTP in the striatum, or alternatively that cells other than forebrain neurons, likely glial cells, may play a role in $A_{2A}$-mediated protection in the striatum (Yu et al., 2008) (Fig. 2).

Studies of toxin-induced striatal neuron death modelling the core neurodegenerative features of Huntington disease rather than PD have also given opposing results regarding the role of $A_{2A}$ receptors. In corticostriatal slices $A_{2A}$ receptor antagonism reduced the irreversible functional alterations caused by rotenone in striatal neurons, supporting a beneficial role of neuronal $A_{2A}$ receptor blockade against neurotoxic insults (Belcastro et al., 2009). By contrast, Huang et al. reported that in a model of acute striatal neuron damage produced by local infusion of the mitochondrial toxin 3-nitropropionic acid (3-NP), selective deletion of $A_{2A}$ receptors on forebrain neurons was unable to protect against neurotoxicity (Huang et al., 2006). In this model global $A_{2A}$ receptor inactivation actually exacerbated 3-NP-induced neurotoxicity. Moreover, the cell-type-specific inactivation of $A_{2A}$ receptors located in bone marrow-derived cells also exacerbated striatal damage (Huang et al., 2006). Altogether, these data, while supporting a neuroprotective outcome of neuronal $A_{2A}$ receptor blockade in PD, highlight the complexities of the roles played by $A_{2A}$ receptors, pointing to distinct actions of cell-type-specific receptors in different neurodegenerative conditions.

In neurons, $A_{2A}$ receptors are enriched in the striatum, but also have been described in other basal ganglia nuclei, such as GP, and at lower levels in the substantia nigra (Brooks et al., 2008; Cunha et al., 1994; Johansson et al., 1997; Rosin et al., 1998; Schiffmann et al., 1991).

At the pre-synaptic level striatal $A_{2A}$ receptors are mostly located on glutamatergic terminals where they modulate the efflux of glutamate (Marchi et al., 2002; Melani et al., 2003; Popoli et al., 2002; Tebano et al., 2004) (Fig. 2). It has been extensively demonstrated that antagonism of the $A_{2A}$ receptor protects against ischemic damage and toxin-induced excitotoxicity in the hippocampus or striatum (Jones et al., 1998; Melani et al., 2006; Popoli et al., 2002). Excessive excitotoxic glutamate efflux from the STN to substantia nigral neurons is a component of PD neuropathology, likely contributing to nigral neuron death. Therefore a reduction of glutamate efflux in the substantia nigra, and perhaps in the striatum, might provide a mechanism for protection against dopaminergic degeneration in PD models (Wallace et al., 2007).

It is important to point out that the effect of $A_{2A}$ receptor antagonists on striatal glutamate levels is dependent on the dose and experimental conditions (Tebano et al., 2004). In intact striatum, the $A_{2A}$ antagonist SCH-58261 decreased evoked glutamate efflux at low doses only, whereas it lost this effect at higher doses (Pintor et al., 2001). Moreover local infusion of an $A_{2A}$ antagonist directly into the 6-OHDA-lesioned striatum increased glutamate outflow (Corsi et al., 2003). Tebano and colleagues have shown that in corticostriatal slices $A_{2A}$ antagonists inhibit glutamate outflow, whereas in striatal neurons they amplify excitotoxic mechanisms due to direct NMDA receptor stimulation (Tebano et al., 2004). Therefore, the potential neuroprotective effect mediated by pre-synaptic $A_{2A}$ receptor blockade might emerge specifically at the low-dose range, whereas other mechanisms, likely mediated by post-synaptic $A_{2A}$ receptors, might mask this effect at higher doses. The higher binding affinity displayed by pre- versus post-synaptic $A_{2A}$ receptors supports this concept (Cunha et al., 1996).

An interesting mechanism of adenosine–glutamate interaction that has emerged recently involves cross-talk between adenosine and glial-derived neurotrophic factor (GDNF) receptors, co-localized on striatal glutamatergic nerve endings (Gomes et al., 2009). In rat striatal synaptosomes, GDNF enhanced glutamate release by

13% from corticostriatal terminals, an effect potentiated by $A_{2A}$ agonist CGS21268 and prevented by the $A_{2A}$ receptor antagonist SCH58261 (Gomes et al., 2009). Therefore, it is suggested that $A_{2A}$ receptor blockade would impair GDNF-stimulated increase of corticostriatal glutamate release, providing a beneficial effect on neurodegeneration (Gomes et al., 2009).

### *Glial $A_{2A}$ receptors and neuroprotection*

Neuroinflammation, characterized by reactive astrocytes and activated microglia, is a hallmark of PD and may play a pathogenic role in dopamine neuron degeneration. Classical studies have described activated microglia in the substantia nigra of PD patients, whereas most recent studies have reported a more widespread distribution of activated microglia in the brain, both in early and in late stages of the disease, involving pons, basal ganglia, striatum and frontal and temporal cortex (Gerhard et al., 2006; Mc Geer and McGeer, 2008). Persistent microglial activation leads to elevated levels of glial-derived cytokines and chronic neuroinflammation, which exert neurotoxic effects on highly vulnerable dopaminergic neurons. On the basis of this evidence it appears of great importance that, in addition to neurons, adenosine $A_{2A}$ receptors are located on both microglia (Fiebich et al., 1996; Saura et al., 2005) and astrocytes (Lee et al., 2003) (Fig. 2). Recent reports *in vivo* have shown that $A_{2A}$ receptor antagonists were able to prevent the astroglial and microglial activation induced by acute or sub-chronic administration of MPTP in mice (Carta et al., 2009; Pierri et al., 2005). These results are in line with that obtained in an *in vivo* model of ischemia, where the specific $A_{2A}$ receptor antagonist SCH58261 inhibited phosphorylation of P38-MAPK (mitogen-activated protein kinase), supporting an inhibitory effect of $A_{2A}$ receptor antagonism on the response in microglial cells (Melani et al., 2006). Therefore, an effect on glial cells has been considered as a potential mechanism of neuroprotection by $A_{2A}$ receptor antagonists. However, the effect on glial response observed in different models of neurodegeneration might either result from a direct effect through stimulation of $A_{2A}$ receptors on these cells, or might be secondary to an effect mediated by $A_{2A}$ receptors on neurons.

The molecular effects of glial $A_{2A}$ receptor stimulation have been investigated by a number of *in vitro* studies, leading to somewhat contradictory results and suggesting that receptors located on microglia and astroglial cells might play opposing roles in neurodegeneration. Supporting a beneficial effect of glial $A_{2A}$ receptor blockade, $A_{2A}$ receptor agonist CGS21680 potentiated lipopolysaccharide (LPS)-induced nitric oxide (NO) release and NO synthase-II expression by microglial cells in a concentration-dependent manner, whereas an $A_{2A}$ antagonist suppressed this effect (Saura et al., 2005). Accordingly, $A_{2A}$ receptors mediated the induction of cyclooxygenase-2 and NO synthase in microglia (Fiebich et al., 1996, 1998).

However, $A_{2A}$ receptor agonists inhibited cytokines production by activated microglia (van der Putten et al., 2009). In cultured astroglia, both adenosine kinase inhibitors and the $A_{2A}$ receptor agonist CGS21280 counteracted LPS-induced production of NO (Brodie et al., 1998; Lee et al., 2005). In line with a positive role of $A_{2A}$ receptor blockade, the $A_{2A}$ receptor agonist CGS21680 increased the astrocytic release of glutamate via an $A_{2A}$ receptor/PKA signalling pathway and via inhibition of glutamate transporter glutamate transporter-1 (GLT-1), resulting in increased synaptic concentrations of this neurotransmitter and likely in deleterious effects on neurons survival. Most importantly, this effect was inhibited by the $A_{2A}$ receptor antagonist DMPX (Nishizaki, 2004). Furthermore, adenosine and the $A_{2A}$ receptor agonist 5′-(*N*-cyclopropyl)-carboxamidoadenosine (CPCA) increased astrocyte proliferation after brain injury in rat cortex, whereas adenosine antagonists where shown to counteract astrocytic proliferation in both *in vitro* and *in vivo* models of brain injury (Brambilla et al., 2003; Hindley et al., 1994; Pugliese et al., 2009).

Therefore, whereas substantial evidence suggests that manipulating glial $A_{2A}$ receptors might be beneficial in neurodegenerative conditions, their selective stimulation or blockade might be beneficial depending on neurodegenerative conditions and time of intervention (Fig. 2). Moreover, reports on glial $A_{2A}$ receptors manipulation in *in vivo* PD models are currently lacking.

Lately, a new alternative mechanism of neuroprotection has been suggested, involving $A_{2A}$ receptors located on oligodendrocytes. In a stroke model $A_{2A}$ receptor antagonist SCH58261 reduced striatal Olig 2 transcription factor induced by ischemia (Melani et al., 2009).

## Epidemiology and clinical trials of adenosine antagonists

### *Caffeine epidemiology*

#### *Case control studies: coffee*

Considerable epidemiological and laboratory data have suggested that $A_{2A}$ receptor blockade by caffeine, a non-selective adenosine receptor antagonist, may protect against the underlying neurodegeneration of PD. Drinking caffeinated beverages (coffee and to a lesser extent tea) has emerged as the dietary factor most consistently linked to an altered risk of PD, with greater consumption associated with a reduced risk (Ascherio et al., 2001; Benedetti et al., 2000; Checkoway et al., 2002; Fall et al., 1999; Hellenbrand et al., 1996; Hu et al., 2007; Ragonese et al., 2003; Ross et al., 2000; Tan et al., 2003). In the early 1990s case–control studies suggested a reduced risk of developing PD associated with drinking coffee but the reduction either was not statistically significant (Jimenez-Jimenez et al., 1992; Morano et al., 1994) or was significant but partially attributable to the confounding association of smoking in coffee drinkers (Grandinetti et al., 1994) and thus difficult to interpret. Following on from this, larger, case–control studies, using better-matched cohorts, demonstrated that even after adjusting for tobacco smoking and other potential confounding factors, the significant inverse relationship between prior coffee drinking exposure and PD remained (Benedetti et al., 2000; Fall et al., 1999; Hellenbrand et al., 1996). These latter studies also specifically investigated dose–response relationships, with increasing coffee consumption (measured in cups per day) associated with decreasing likelihood of having developed PD. In these retrospective analyses PD patients were 4–8 times less likely than control subjects to have reported being heavy coffee drinkers in the past. While case–control studies have some advantages, weaknesses of these designs for investigating dietary aetiology of chronic diseases are well known (Willett, 1998) and include, for example, difficulty selecting appropriate control subjects and recall bias. The introduction of large cohort prospective investigations (see below) has overcome many of these limitations through follow-up evaluations to determine disease incidence in subjects from a single population in which the exposure in question (i.e. coffee or caffeine consumption) had been reported years earlier.

#### *Case–control studies: tea*

Relatively few studies have considered the effect of tea drinking and its association with PD risk. This could be attributed to its low consumption in North America and Europe (Ascherio et al., 2001; Chan et al., 1998). These studies usually (Ayuso-Peralta et al., 1997; Chan et al., 1998; Checkoway et al., 2002; Hellenbrand et al., 1996; Ho et al., 1989; Tan et al., 2003, 2007), although not always (Preux et al., 2000), indicated a reduced risk of developing PD amongst frequent tea drinkers. Interestingly, epidemiological studies from China have observed that the prevalence of PD is much lower than in the Caucasian population (Li et al., 1985; Zhang and Roman, 1993). Barranco et al., 2009 reviewed observational studies that evaluated tea consumption and the risk of PD (11 case–control

and 1 cohort) between 1981 and 2003. The studies represented documented cases from North America, Europe and Asia. Amongst the case–control studies, the pooled OR was 0.8 (95% CI 0.71–0.90) suggesting that tea consumption is inversely associated with the risk of PD. It is unclear whether the active ingredient(s) mediating this observed protective effect is caffeine or some biologically active substance(s) present in tea but not coffee.

### *Cohort studies*

#### *Caffeinated beverages*

More convincing epidemiological evidence that caffeine and coffee consumption are linked to a reduced risk of developing PD has been obtained from the study of prospectively followed large populations (Ascherio et al., 2001; Ross et al., 2000). Three decades after ~8000 Japanese-American men were enrolled in the Honolulu Heart Program (and provided details of their dietary caffeine consumption), over 100 had gone on to develop PD. Higher initial coffee intake was dose dependently associated with a reduced incidence of PD, with a fivefold lower risk amongst those who drank over 24 oz per day (Ross et al., 2000). Confirmation of these findings was provided by two prospective studies of larger, multiethnic populations, namely the Health Professionals Follow-up Study (HPFS) of 50 000 men followed for a period of 10 years and the Nurses Health Study (NHS) of 90 000 women followed over 16 years (Ascherio et al., 2001). Amongst the men, increased coffee, tea and non-coffee caffeine consumption and total caffeine consumption were all significantly, dose dependently and negatively correlated with the incidence of subsequent PD. These associations were independent of smoking and other potential confounding factors. By contrast, the rates of consuming *decaffeinated* coffee were unrelated to the risk of PD, implicating caffeine as the component in coffee that is inversely associated with PD risk. Similar findings were observed in two separate cohort studies of Finnish men and women free from PD at baseline (Hu et al., 2007; Saaksjarvi et al., 2008). Heavier coffee consumption was associated with a reduced risk of PD even after adjustment for confounding factors. The Finnish population is of particular interest since it exhibits one of the world's highest rates of coffee consumption (Fredholm et al., 1999).

#### *Gender differences (oestrogen interactions)*

Interestingly, a stark gender difference in how caffeine relates to PD risk emerged from comparison of female and male cohorts within a large protective study of health care workers (Ascherio et al., 2001). Initially, analysis of women in the NHS study revealed no clear relationship between PD and caffeine or coffee intake. This gender difference was consistent with the observations of PD incidence rates in Olmstead County, MN, which were strongly inversely related to prior coffee drinking in men (with a ~17-fold reduction in risk amongst drinkers vs. non-drinkers; $p < 0.01$) but did not vary with coffee exposure in women (with a relative risk of 1.0 ; Benedetti et al., 2000).

Ascherio and colleagues gained insight into stratification of the women by oestrogen exposure history. In two separate prospective studies (Ascherio et al., 2003, 2004) they showed that amongst women who did not use post-menopausal oestrogens, caffeine was in fact associated with a reduction in the risk of subsequent PD (just as in men). Conversely, for women who had used oestrogen replacement caffeine use did not carry a lower risk of PD, suggesting a hormonal basis for the gender difference in caffeine's association with PD.

Interestingly, in contrast to the result from studies by Ascherio and colleagues, the two prospective cohort studies of Finnish populations (Hu et al., 2007; Saaksjarvi et al., 2008) reported no gender differences in the (inverse) relationship between coffee consumption and PD risk. However, as Saaksjarvi and colleagues note in their study the effect of post-menopausal hormone use

could not be examined due to small number of users, which was reported to be ~5% of women and markedly less than in the US cohorts. For example, in the NHS, a third of the women were currently taking post-menopausal oestrogens and 54% reported ever taking them. Thus, the variability in an overall association between caffeine and PD risk among women between cohorts may be explained by wide variation in their use of post-menopausal oestrogen, which appears to complicate the relationship between caffeine and PD.

Recent laboratory experiments have explored the biology that may underlie gender differences in the caffeine–PD link observed in populations with higher rates of oestrogen use. Xu et al. (2006) found that the ability of caffeine to attenuate MPTP toxicity in a mouse model of PD was greater in male versus female mice and in ovariectomized versus sham-operated female mice. They also demonstrated that chronic oestrogen replacement undermined caffeine's protective effect both in male and in ovariectomized female mice, providing direct evidence of a hormonal influence on caffeine's neuroprotective properties in lab animals. The study also implicated a biological basis for the gender difference in the association between caffeine consumption and PD risk. In general, these and other laboratory studies (as reviewed above) support – but do not prove – the hypothesis that the consistent epidemiological association between caffeine and a reduced risk of PD is causal.

*PD progression*

Convergent epidemiological and laboratory data support the possibility that dietary caffeine reduces the risk of developing PD. Less well known is the relationship between caffeine intake and the rate of progression of the disease. A preliminary investigation (Schwarzschild et al., 2003a) reported a secondary analysis of the 'Comparison of the agonist pramipexole versus L-DOPA on the motor complications of PD' (CALM-PD) trial database, which included data over 5 years from 268 participating PD patients (87% of the original CALM-PD cohort). No association was found between caffeine intake (from coffee, tea and soda sources) and rate of PD progression even after adjustment for treatment group, gender, tobacco and alcohol use. Outcome measures assessed progression by change in the Unified Parkinson Disease Rating Scale (UPDRS) or by alteration in the striatal uptake of iodine-123-labeled 2-β-carboxymethoxy-3-β-(4-iodophenyl) tropane ([$^{123}$I] β-CIT) in the subset of patients that underwent neuroimaging. The study also allowed for assessment of caffeine consumption and dyskinesia development. A trend albeit non-significant towards lower risk of dyskinesias with increasing caffeine intake was observed. The lack of an association between caffeine consumption and rate of subsequent progression was also observed in a NET-PD (NIH Exploratory Trials in Parkinson's Disease) trial cohort (Simon et al., 2008) monitoring changes in the UPDRS score and likelihood of disease progression to the point of requiring symptomatic therapy as measurable outcomes. These studies do not lend support to a protective benefit of caffeine by PD patients. However, they leave open the possibility that disease progression among individuals who develop PD despite ingesting caffeine at higher levels may be less influenced by any true protective effect it may have.

## Clinical trials of $A_{2A}$ antagonists for PD

### *Trials for symptomatic anti-parkinsonian indications*

*Non-selective adenosine antagonists: caffeine and theophylline*

Although caffeine is a non-specific adenosine receptor antagonist that blocks with similar potency at the $A_1$, $A_{2A}$ and $A_{2B}$ sub-types (Fredholm et al., 1999), it appears to have its main psychomotor stimulant actions primarily through blockade of CNS $A_{2A}$ receptors (Xu et al., 2004;

Yu et al., 2008). Thus the first trials of $A_{2A}$ antagonism in PD may have been a pair of double-blind, placebo-controlled studies of caffeine effects on parkinsonian symptoms of patients taking L-DOPA or a dopamine agonist in the 1970s (Kartzinel et al., 1976; Shoulson and Chase, 1975). Both studies found no benefit of caffeine. However, their small size (<10 subjects), clinical heterogeneity (some subjects did not have PD) and extremely high dosing 1–1.5 g of caffeine daily precludes meaningful conclusions about the symptomatic utility of caffeine for PD from these studies. A more rigorously designed randomized, placebo-controlled trial of caffeine at more pharmacologically informative doses (100 or 200 mg bid) is currently underway and projects enrollment of some 50 subjects with idiopathic PD and excessive daytime somnolence (McGill University Health Center; clinical trials.gov identifier # NCT00459420). Although its primary outcome focuses on daytime sleepiness, a secondary analysis of parkinsonian motor symptoms is planned using the standard UPDRS.

Theophylline, a demethylation metabolite of caffeine as well as an anti-asthmatic agent in common use, is also a non-specific adenosine antagonist. Although small open-label trials of theophylline in PD seemed to suggest anti-parkinsonian benefit without exacerbation of dykinesias (Kostic et al., 1999; Mally and Stone, 1994), a subsequent double-blind, placebo-controlled trial of theophylline in PD did not clearly demonstrate relief from symptoms (Kulisevsky et al., 2002). However, the power and design of all these trials like the earlier caffeine studies in PD were not sufficient to rule out clinically useful symptomatic effects, as have been suggested by long-standing preclinical studies of motoric enhancement in PD models (Stromberg and Waldeck, 1973).

### *Selective adenosine $A_{2A}$ antagonists*

A clear indication of the promise of adenosine $A_{2A}$ receptor antagonism for PD is the depth of industry investment in programs to develop a variety of xanthine and non-xanthine structure-based adenosine receptor antagonists as anti-parkinsonian agents. Amongst at least 10 publicly announced programs are some 5 that have entered human studies, with 4 being now actively pursued through phase II and III clinical trials: istradefylline (aka KW-6002) from Kyowa-Kirin (Kyowa Hakko Kirin Co., 01.15.2009 announcement), Preladenant (aka SCH 420814) from Schering Plough (now Merck) (Schering Plough Co., 11.24.2008 announcement), BIIB014 (aka V2006) from BiogenIdec (licensed from Vernalis) (Papapetropoulos et al., 2010) and Syn-115 from Synosia (licensed from Roche) (Black et al., 2010a, 2010b).

The early frontrunner amongst these clinical development programs was istradefylline, which had progressed through encouraging phase II trial results (Bara-Jimenez et al., 2003; Fernandez et al., 2010; Hauser et al., 2003, 2008; Kase et al., 2003; Lewitt et al., 2008; Stacy et al., 2008) to as yet unpublished, reportedly mixed results of phase III studies (Kyowa Hakko Kirin Co., 06.03.2007 announcement) before submission of a New Drug Application (NDA) to US Food and Drug Administration (FDA). However, the FDA in response issued a 'Non-Approvable letter' in 2008 based on concerns of the adequacy of overall efficacy (Kyowa Hakko Kirin Co., 02.08.2008 announcement). Since then clinical development of istradefylline has continued, but the company has pulled back from an international program to focus on trials and a potential indication in Japan.

Based on available data for the published phase II trials, istradefylline employed (at once-daily doses from 20 to 80 mg) as adjunctive therapy in relatively advanced subjects, produced a modest but significant reduction in 'off' time (i.e. in motor dysfunction). Of note, these 'positive' outcome measures were based on reports of the patients themselves, whereas no significant treatment-induced improvement was observed based on the clinician-scored UPDRS. Although preclinical evidence supporting the original study designs had suggested adjunctive $A_{2A}$ antagonism might

assuage PD symptoms without exacerbating dyskinesias [reviewed in Xu et al. (2004)], istradefylline treatment in this relatively advanced PD population actually increased dyskinesias in most studies. However, when dyskinesias were stratified into 'troublesome' and 'non-troublesome', only the latter were significantly increased on the drug.

Recently, istradefylline was also tested as monotherapy for its potential symptomatic benefit in early PD (Fernandez et al., 2010). Despite a trend towards improvement on istradefylline, again the difference between placebo and $A_{2A}$ antagonist-treated groups did not reach significance for the change in UPDRS score, the primary outcome in this study.

Retrospective assessment of why this early $A_{2A}$ antagonist development program fell short in its initial efforts to gain an indication for PD treatment has focused on trial design elements, including dose selection (for both istradefylline and concomitant L-DOPA) and disease stage of the targeted PD subpopulation (i.e. possibly too advanced in adjunctive trials, though possibly too early in the monotherapy trial).

In addition, none of the trial reports addressed the potential confound of concomitant $A_{2A}$ antagonism by caffeine use, which apparently was neither excluded nor monitored. At doses relevant to typical human consumption, caffeine and more specific $A_{2A}$ antagonists (including istradefylline) bind to striatal $A_{2A}$ receptors *in vivo* to a similar extent (El Yacoubi et al., 2001; Moresco et al., 2005). Given also the well-known psychomotor stimulant properties of caffeine and its continued consumption in PD with a mean intake of approximately 200 mg/day among early in the disease (Schwarzschild et al., 2003a; Simon et al., 2008), its use is important to consider in $A_{2A}$ antagonist trials. As caffeine use tends to decrease over the course of the disease, controlling for its use in the analysis of early PD/monotherapy trials may be particularly informative. If specific adenosine $A_{2A}$ receptor antagonists were found to be more effective amongst those consuming less caffeine (i.e. less of a general adenosine antagonist), it would support the distinct possibility of a shared anti-parkinsonian effect through a common mechanism. It would then remain to be determined whether specific $A_{2A}$ receptor antagonism offers an advantage of greater efficacy or tolerability (given possibly adverse effects of blocking other adenosine receptors) to offset the lower cost and greater availability of caffeine.

### *Trials for disease modification in PD*

Convergent epidemiological and laboratory studies have suggested that adenosine $A_{2A}$ antagonism may confer disease-modifying benefits beyond the anti-parkinsonian motor effects now being actively pursued in clinical trials as above (Schwarzschild et al., 2006; see Fig. 3). Although caffeine itself was listed amongst the most attractive candidate neuroprotectants under consideration for clinical investigation (Ravina et al., 2003), if a specific $A_{2A}$ antagonist were to receive an indication for symptomatic relief, it may be more likely than caffeine to first undergo testing in a 'neuroprotection' trial given the high costs of conducting the necessarily large and long-term trials required.

The prospects of favourable disease modification by $A_{2A}$ receptor blockade may extend beyond its neuroprotective potential as $A_{2A}$ receptors have also been implicated in the maladaptive neuroplasticity that underlies the development of L-DOPA-induced dyskinesia (LID) in PD (Morelli et al., 2009). Accordingly, early treatment with an $A_{2A}$ antagonist versus placebo as an adjunct to newly initiated L-DOPA may also be considered as a novel trial design to assess for both an early synergistic benefit (possibly with greater sensitivity than a monotherapy design) and a prophylactic effect on LID during a second long-term phase of observation with subjects maintained in their blinded treatment arms.

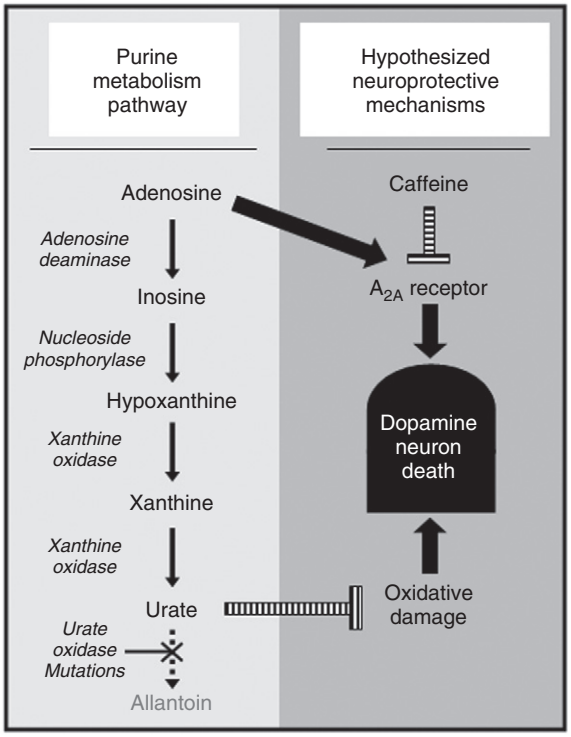


Fig. 3. Therapeutic targets along the purine metabolic pathway. Adenosine $A_{2A}$ antagonists (including caffeine) and urate have emerged as realistic candidate neuroprotectants. In humans the enzymatic metabolism of purines such as adenosine ends with urate due to multiple mutations within the *urate oxidase* gene during primate evolution (see text). The schematic suggests a possible homeostatic mechanism linking an adenosinergic neurodegenerative influence with an offsetting neuroprotective influence of urate.

## Urate as a novel target for neuroprotection

### *Biology*

#### *Evolutionary significance*

During primate evolution a series of mutations occurred in the urate oxidase gene (*UOx*) likely accounting for the relatively high levels of urate (the physiologically dissociated form of uric acid) in apes and humans compared to other mammals (Oda et al., 2002; see Fig. 3). Urate is not only the end product of the metabolism of purines like adenosine. It also possesses antioxidant properties comparable to those of ascorbate (Ames et al., 1981) and accounts for most of the antioxidant capacity in human plasma (Yeum et al., 2004), supporting the hypothesis that our ancestors gained antioxidant benefits from *UOx* mutations and a resulting elevation of urate concentrations (Proctor, 1970). While higher levels of urate may have conferred an evolutionary advantage through bolstered defences against oxidative damage (Ames et al., 1981) today they are also the core molecular culprit in gout and uric acid kidney stones.

#### *Neuroprotective effects in cellular models of PD*

In addition to its antioxidant actions, urate has also been shown to possess other potentially protective properties *in vitro*. It has been shown to scavenge peroxynitrite as well as oxygen free radicals *in vitro* (Franzoni et al., 2006; Whiteman et al., 2002) and displays potent iron-chelating activity independent of its direct antioxidant action (Davies et al., 1986). Direct evidence for a neuroprotective effect of urate has come initially from cellular and animal models of multiple sclerosis (Hooper et al., 1997, 1998; Scott et al., 2002), stroke (Romanos et al., 2007; Yu et al., 1998) and spinal cord injury (Du et al., 2007; Scott et al., 2002).

Urate also confers protection in cellular models of PD. In PC12 cells, urate blocked apoptosis and oxidant production induced by dopamine (Jones et al., 2000) or the pesticide rotenone in combination with homocysteine (Duan et al., 2002) and reduced cell death induced by MPP+ or $Fe^{2+}$ (Haberman et al., 2007). Urate also attenuated toxin-induced loss of primary neurons in culture. A recent study of dopaminergic neurons in primary midbrain culture of rat ventral mesencephalon found that their physiological function and survival were significantly enhanced by urate (Guerriero et al., 2009) at concentrations ($\geq$30–50 μM)

corresponding to those in human cerebrospinal fluid (CSF) associated with a reduced rate of clinical decline in PD (Ascherio et al., 2009).

### *Epidemiology*

When first considered in case–control studies, lower urate levels were found in serum (Andreadou et al., 2009; Annanmaki et al., 2007; Bogdanov et al., 2008; Johansen et al., 2009; Larumbe et al., 2001), possibly in cerebrospinal fluid (Tohgi et al., 1993), and in post-mortem nigrostriatal tissue samples (Church and Ward, 1994) of PD patients compared to those of controls. These studies suggested that low CNS and peripheral levels of urate are associated with PD.

A series of epidemiological investigations of prospectively followed cohorts has more incisively linked higher blood urate with a reduced risk of developing PD (Chen et al., 2009; Davis et al., 1996; De Lau et al., 2005; Weisskopf et al., 2007). For example, in the largest of these cohorts, 18 000 men were followed for more than 8 years in the HPFS. Weisskopf and colleagues found that those in the top quartile of plasma urate concentration had a two to threefold lower risk of PD than subjects in the bottom quartile ($p < 0.02$ for trend across all quartiles). Amongst the subset of cases for whom blood was collected at least 4 years before the diagnosis of PD, an even greater reduction of PD risk was observed – with those in the highest urate quartile having a fivefold lower risk of PD compared to the lowest quartile ($p < 0.01$ for trend). This further analysis suggests that the low uricemia among individuals with PD precedes the onset of neurological symptoms and is thus unlikely to be a consequence of changes in diet, behaviour or medical treatment early in the course of the disease. This inverse association was independent of age, smoking, caffeine consumption and other aspects of lifestyle that have been related to PD or uricemia.

Similarly, urate-elevating diet was also associated with a lower risk of PD (Gao et al., 2008). In the HPFS cohort, the authors found that a higher dietary uricemic index (reflecting dietary patterns linked to higher plasma urate) predicted a reduced risk of developing PD ($p < 0.001$ for trend). The association between the index and the PD risk remained strong and significant in models further adjusted for age, smoking and caffeine intake. Their findings suggest that dietary interventions that raise blood urate concentrations might reduce the risk of PD.

In a related set of epidemiological studies of large prospectively followed cohorts, a diagnosis of gout (a form of arthritis due to urate crystallization in joints and associated with hyperuricemia) was linked to a lower risk of later being diagnosed with PD (Alonso et al., 2007; De Vera et al., 2008). Together these epidemiological data establish urate exposure – assessed by laboratory, dietary or pathological indicators – as a robust inverse risk factor for PD.

### *Clinical studies of urate and PD progression*

The emergence of robust epidemiological data linking higher urate levels amongst healthy populations to a reduced risk of developing PD prompted a corollary hypothesis: *Amongst people already diagnosed with PD, do higher urate levels predict a slower rate of clinical decline?* To test the hypothesis, incidentally measured urate levels in two large completed 'neuroprotection' trials were related to rates of clinical decline over years. Although neither the PRECEPT (Parkinson Study Group, 2007) nor the DATATOP (Parkinson Study Group, 1993) trial had demonstrated efficacy of candidate neuroprotectants, each had collected data for routine safety lab tests – including serum urate – at enrollment of some 800 recently diagnosed '*de novo*' PD patients, who were then followed closely for 2 years. For both trials the primary outcome was time to disability warranting the initiation of L-DOPA or dopaminergic agonist therapy, with secondary outcomes including rate of UPDRS change and, in the case

of the PRECEPT trial, rate of loss of dopamine transporter (DAT) ligand uptake in the striatum.

In the PRECEPT cohort, subjects with higher (but still normal) levels of serum urate at baseline were significantly less likely to develop disability warranting dopaminergic therapy and also retained significantly more striatal DAT binding capacity during the study (Schwarzschild et al., 2008). For example, subjects in the top quintile of serum urate (~7–8 mg/dl with normal value reference ranges typically 3–8 mg/dl) reached the end point at only half the rate of subjects in the lowest quintile (hazard ratio, 0.51; 95% confidence interval, 0.37–0.72; $p$ for trend <0.001). In the DATATOP cohort higher baseline urate concentrations in CSF as well as in serum were similarly associated with a slower rate of reaching the primary disability end point (Ascherio et al., 2009). In both cohorts higher urate levels were also predictive of a favourable rate of clinical decline measured by the change in UPDRS score. Although several descriptive clinical features of PD have been identified as probable predictors of the rate of clinical decline in PD (Post et al., 2007), urate may be the first molecular factor clearly linked to clinical progression of idiopathic PD.

In addition to its emerging utility as a prognostic biomarker of PD in research studies, the demonstrated antioxidant and neuroprotective properties of urate raise the possibility of its potential for direct therapeutic benefit. Convergence of these biological, epidemiological and clinical findings has prompted rapid translation to clinical application (Parkinson Study Group; clinical trials.gov identifier # NCT00833690). A multi-centre, randomized, placebo-controlled trial of inosine (a precursor of urate as well as the deamination product of adenosine in purine metabolism; see Fig. 3) in early PD is currently underway to assess its safety and ability to elevate serum and CSF urate levels and its potential for further development as a novel strategy to impede progression of the disease.

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**Abbreviations**

| | |
|---|---|
| 3-NP | 3-nitropropionic acid |
| 6-OHDA | 6-hydroxydopamine |
| BRET | Bioluminescence Resonance Energy Transfer |
| cAMP | cyclic adenosine monophosphate |
| CNS | central nervous system |
| CPX | 8-cyclopentyl-1,3-dipropylxathine |
| CREB | cAMP response element-binding |
| DARPP-32 | dopamine- and cAMP-regulated phosphoprotein-32 |
| DAT | dopamine transporter |
| ERK | Extracellular signal-regulated kinases |
| FDA | US Food and Drug Administration |
| FRET | fluorescence resonance energy transfer |
| GABA | gamma-aminobutyric acid |
| GDNF | glial-derived neurotrophic factor |
| GLT-1 | glutamate transporter-1 |
| HPFS | Health Professionals Follow-up Study |
| L-DOPA | L-3,4-dihydroxyphenylalanine |
| LID | L-DOPA-induced dyskinesia |
| LPS | lipopolysaccharide |
| MAPK | mitogen-activated protein kinase |
| MPTP | 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine |
| NDA | New Drug Application |
| NGF | nerve growth factor |

| | |
|---|---|
| NHS | Nurses Health Study |
| NO | nitric oxide |
| PD | Parkinson's disease |
| STN | subthalamic nucleus |
| TH | tyrosine hydroxylase |
| UPDRS | Unified Parkinson's Disease Rating Scale |

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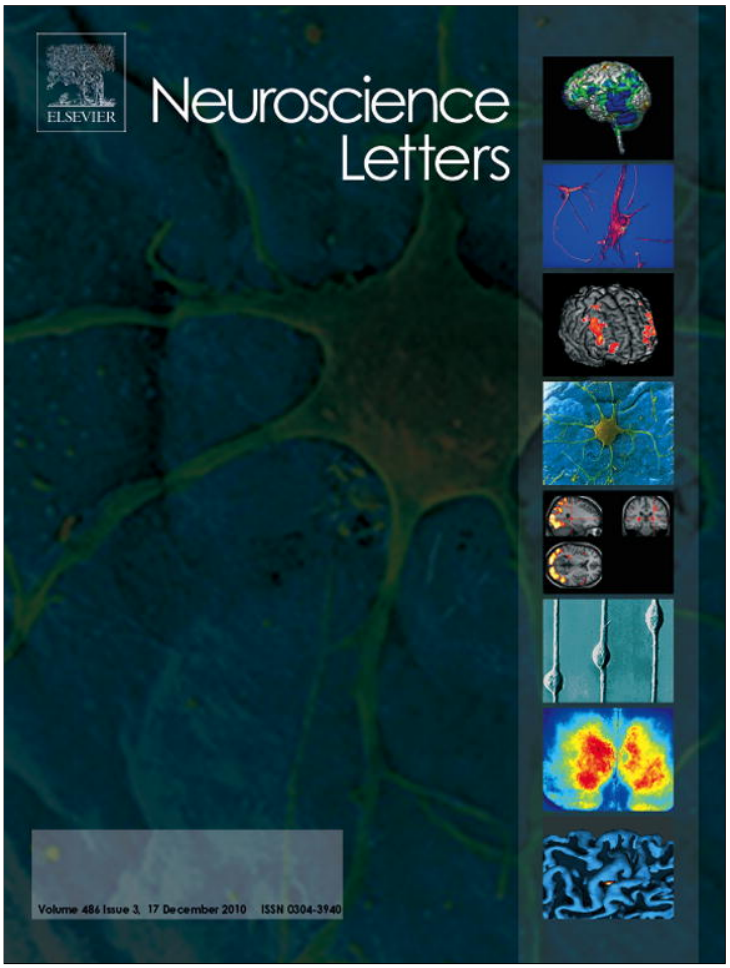
Appendix V



Appendix V

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# Protective effect of metabotropic glutamate mGluR5 receptor elimination in a 6-hydroxydopamine model of Parkinson's disease

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ABSTRACT

Pharmacologic or genetic blockade of metabotropic glutamate mGlu5 receptors (mGluR5) has been shown to attenuate parkinsonian motor deficits and protect nigrostriatal neurons from damage in the acute MPTP model of Parkinson's disease (PD), suggesting that therapeutically targeting the mGluR5 receptor may offer a novel approach to improving motor symptoms and/or slowing neurodegeneration in PD. This study further explored the neuroprotective potential of targeting mGluR5 receptors. We examined the behavioral and neurochemical effects of receptor elimination on toxicity induced by intra-striatal application of 6-hydroxydopamine (6-OHDA), thought to represent a comparatively progressive model of PD. mGluR5 knockout (KO) mice and wild-type (WT) littermates received unilateral 6-OHDA infusions. Reflecting the imbalance expected following unilateral infusion, WT but not KO mice demonstrated predominantly ipsilateral forepaw use and robust ipsilateral amphetamine-induced rotation. Further, performance on the vertical pole descent task was profoundly impaired in WT mice, while KO mice completed the task significantly faster. Consistent with the behavioral observations, neurochemical analyses of striatal dopamine depletion showed significantly diminished severity in KO mice with only 64% of striatal dopamine lost, compared to 92% in WT mice. The absence of brain mGluR5 receptors in living KO mice was verified using positron emission tomography (PET). Our findings substantiate the key role of mGluR5 receptors in animal models of PD, strengthening the rationale for the development of mGluR5 antagonists for their neuroprotective, as well as symptomatic, benefit.



Parkinson's disease (PD) patients have markedly reduced levels of striatal dopamine (DA) due to the progressive degeneration of substantia nigra *pars compacta* (SNpc) neurons, resulting eventually in debilitating motor dysfunction. Although partial relief of motor symptoms can be achieved primarily with 'DA replacement' therapies, no approach has been established as a means to alter the underlying neurodegenerative process. Therefore, investigations to identify new treatment strategies that may slow the progression of PD continue to be a priority.

A potentially efficient approach to pursuing protective drug candidates is to target non-dopaminergic transmitter systems that may impact both degenerative and motor pathophysiology of the basal ganglia (for example, [5,9,11,13,33]). Of interest due to its interaction with the dopaminergic system, metabotropic glutamate mGluR5 receptors are located on striatal neurons [24,34,36] and modulate excitatory glutamatergic transmission (for reviews, see [8,26]), thereby counterbalancing dopaminergic input from the SNpc. The progressive loss of substantia nigra dopaminergic neurons in PD may result in the overstimulation of glutamatergic receptors in striatal output pathways, contributing to PD pathophysiology. Blockade of mGluR5 function with antagonist drugs such as 2-methyl-6-(phenylethynyl)pyridine (MPEP) can attenuate parkinsonian motor deficits [1,9,18] and decrease the abnormal involuntary movements (dyskinesias) that develop following chronic levodopa treatment in animal models of PD [14,17,20,29].

Blockade of mGluR5 may also protect against neurodegeneration. Research utilizing *in vivo* and *in vitro* models of brain trauma, excitotoxicity, and stroke has indicated that mGluR5 antagonists can be neuroprotective [3,21,27]. Further, in animal models of PD, mGluR5 antagonists lessen nigrostriatal degeneration [2,4,37,38]. In addition, Battaglia et al. showed that elimination of the mGluR5 receptor reduced nigrostriatal neuron damage following exposure to MPTP, a toxin that causes an acute, non-progressive loss of DA neurons [4].

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The present investigation further explores the protective potential of mGluR5 elimination using a relatively progressive model of PD neurodegeneration, in which striatal 6-OHDA infusion leads to the gradual loss of nigrostriatal DA neurons ([32] for review; [6,23,28,31]). This toxin model allowed us to compare behavioral and neurochemical profiles of mGluR5 receptor knockout (KO) and wild-type (WT) mice over a period of 4 weeks, during which time the retrograde loss of dopaminergic cells in the substantia nigra evolves more slowly than in MPTP models of PD ([32] for review), and thus may be more representative of the degeneration that occurs in humans. We tested the hypothesis that mice lacking the mGluR5 are more resistant to toxin-induced damage, as assessed by DA depletion and motor impairment following intrastriatal 6-OHDA lesioning.

All experiments were performed in accordance with NIH guidelines on the ethical use of animals. As previously described [18], mGluR5 heterozygote KO (+/−) mice were obtained from the Jackson Laboratory (Bar Harbor, ME; B6.129-*Grm5*$^{tm1Rod}$/J, stock #003558), back-crossed until in an incipient (N7) congenic C57BL/6 genetic background, and crossed to generate the PCR-genotyped mGluR5 KO, WT, and heterozygote (HZ) littermates used in this study. Male mice from 4 to 10 months of age were maintained in home cages under a 12 h light/dark cycle, with *ad libitum* access to food and water. Twenty-three mice (12WT/11KO) received infusions of 10 μg of freshly prepared 6-OHDA bromide salt into the left striatum at the following coordinates (mm from bregma: 0.5 A, 2.0 L, 2.8 V) as was previously described [15,40]. HZ littermates were used as unlesioned controls for normal behavioral activity given our prior demonstration that unlesioned homozygous mGluR5 KO mice display normal baseline behaviors indistinguishable from WT littermates (e.g., in open field locomotor testing) [18].

Fourteen days after lesioning surgery, vertical pole and beam traversal tasks were conducted to assess motor coordination and balance. Forepaw asymmetry and amphetamine-induced rotation tests were conducted to assess the extent of motor asymmetry (for review, see [10]) in WT and mGluR5 KO mice compared to unlesioned HZ mice. The asymmetry tasks were conducted at the beginning and end of a 14-day period that began 14 days post-lesion, with both motor coordination assessment tasks performed in the middle of this period. First, we evaluated forepaw asymmetry (10 WT/8 KO), defined as predominate use of the forepaw ipsilateral to the left striatal lesion while rearing. Individual mice were placed in a translucent cylinder and the number of ipsilateral, contralateral, and bilateral (symmetric) forepaw placements were counted for 5 min by an experimenter blind to mouse genotype. The number of ipsilateral forepaw placements were divided by the total number of placements and multiplied by 100 to obtain a percentage ipsilateral forepaw preference.

Next, to determine the extent of motor impairment using the vertical pole descent task, individual mice (10 WT and 8 KO lesioned mice, and 4 HZ unlesioned mice) were placed facing upwards along the top of a 50 cm vertical wooden pole (1 cm diameter). Total descent time, comprised of the time to orient downwards plus the time to descend the pole until all 4 paws were on the support block, was measured. Five consecutive trials were run in 1 day. During the beam traversal task, these mice were placed on 1 m long wooden beam that was divided into 4 sections of widths 3.5, 2.5, 1.5, and 0.5 cm. The beam was supported 50 cm above the table surface. The total time to traverse the beam, as well as slips, falls, and experimenter-facilitated restarts, were recorded during 4 consecutive trials in 1 day.

In a final behavioral assessment, to further quantify asymmetry, rotational response of mice (10 WT/8 KO) to DA agonist amphetamine (2.5 mg/kg i.p.) was measured. Contralateral and ipsilateral rotations were recorded for 60 min as previously described [40].

Four weeks after unilateral 6-OHDA lesioning and 24 h after the last behavioral experiment (amphetamine-induced rotation), all mice were euthanized striata were quickly dissected out for analysis of DA and its metabolite DOPAC by high-performance liquid chromatography (HPLC) coupled to electrochemical detection [12].

Using PET to verify the functional depletion of mGluR5 receptors in KO mice, a separate set of KO and WT mice were imaged *in vivo* with a selective and sensitive mGluR5 antagonist, 3-[$^{18}$F]Fluoro-5-(2-pyridinylethynyl)benzonitrile ([$^{18}$F]FPEB), a highly specific PET tracer for mGluR5 [30,39]. An additional imaging session was also carried out using [$^{11}$C]CFT ([11C]2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane), a sensitive and selective DA transporter (DAT) ligand, to investigate dopaminergic denervation. A total of 9 mice (4 WT/5 KO) were imaged with [$^{18}$F]FPEB and 4 mice (2 WT/2 KO) were imaged with [$^{11}$C]CFT. Imaging studies were performed using a microPET P4 tomograph (Concord Microsystems, Knoxville, TN) as previously described [25]. After imaging was completed, mice were sacrificed, brains were removed and stored at −80° C. Genotype was confirmed by the presence of appropriately sized mGluR5 immunoreactivity in tissue using western blot analysis (data not shown).

Data are expressed as mean ± SEM. Statistical analyses were conducted using two-way ANOVA and Student's *t* test where appropriate. Statistical significance was considered reached when $p < 0.05$. Bonferroni/Dunn tests were used for *post-hoc* analyses.

6-OHDA-induced motor impairment was attenuated by mGluR5 receptor depletion. The lesioned WT mice used significantly longer time (turn time + descent time) to complete the vertical pole descent task across all 5 trials than lesioned KO or unlesioned mice ($F_{2,17} = 6.15$, $P = 0.01$; Fig. 1A). Closer observation revealed that all groups took a similar amount of time to orient downwards on the pole, but lesioned WT mice were significantly slower descending the pole ($F_{2,17} = 6.87$, $P = .007$). Overall, there were no differences between the performance of lesioned KO mice and unlesioned mice on this task (Fig. 1A). On the beam traversal task, lesioned mGluR5 KO and WT mice crossed the beam slower than unlesioned mice ($F_{2,19} = 4.28$, $P = 0.03$; Fig. 1B). Although, lesioned KO mice tended to cross faster compared to WT mice during intermediate trials, no significant difference was appreciated. However, all groups decreased their latency to cross the beam across trials ($F_{3,57} = 16.38$, $P < 0.0001$) and exhibited equivalent numbers of slips, falls, and restarts during the task (data not shown).

As expected after unilateral lesions, WT mice demonstrated profound asymmetry when rearing and making forepaw contact with the cylinder wall. Unilaterally lesioned WT mice used only the ipsilateral forepaw on 82 ± 9% of cylinder wall touches (Fig. 1C; total touch rate of 6.5 ± 1.1 per 5 min), with the remainder being bilateral forepaw touches because no contacts (0.0 ± 0.0%) were made with just their contralateral forepaw. Lesioned mGluR5 KO mice demonstrated less forepaw use asymmetry, touching exclusively with the ipsilateral or contralateral forepaw on 47 ± 12% or 10 ± 4%, respectively, of all wall contacts. Their total touch rate was 10.3 ± 3.0 contacts per 5 min, which was not significantly greater than for their lesioned WT littermates. Likewise, amphetamine induced a greater ipsilateral turning bias (96 ± 2% of 162 ± 37 total turns) in lesioned WT mice, in contrast to a more symmetric response (65 ± 9% of 94 ± 25 total turns) in lesioned mGluR5 mice (Fig. 1D). Thus the asymmetry was significantly greater in lesioned WT compared to mGluR5 KO mice on both the forepaw asymmetry ($t_{14} = 2.33$; $P = 0.035$, Student's *t* test and $P = 0.0155$, nonparametric Mann–Whitney test) and amphetamine rotation ($t_{16} = 3.93$; $P = 0.001$) tests.

These data were substantiated by findings from post-mortem HPLC analyses of striatal DA and DOPAC. By comparing residual striatal DA/DOPAC content from the 6-OHDA-lesioned left hemi-

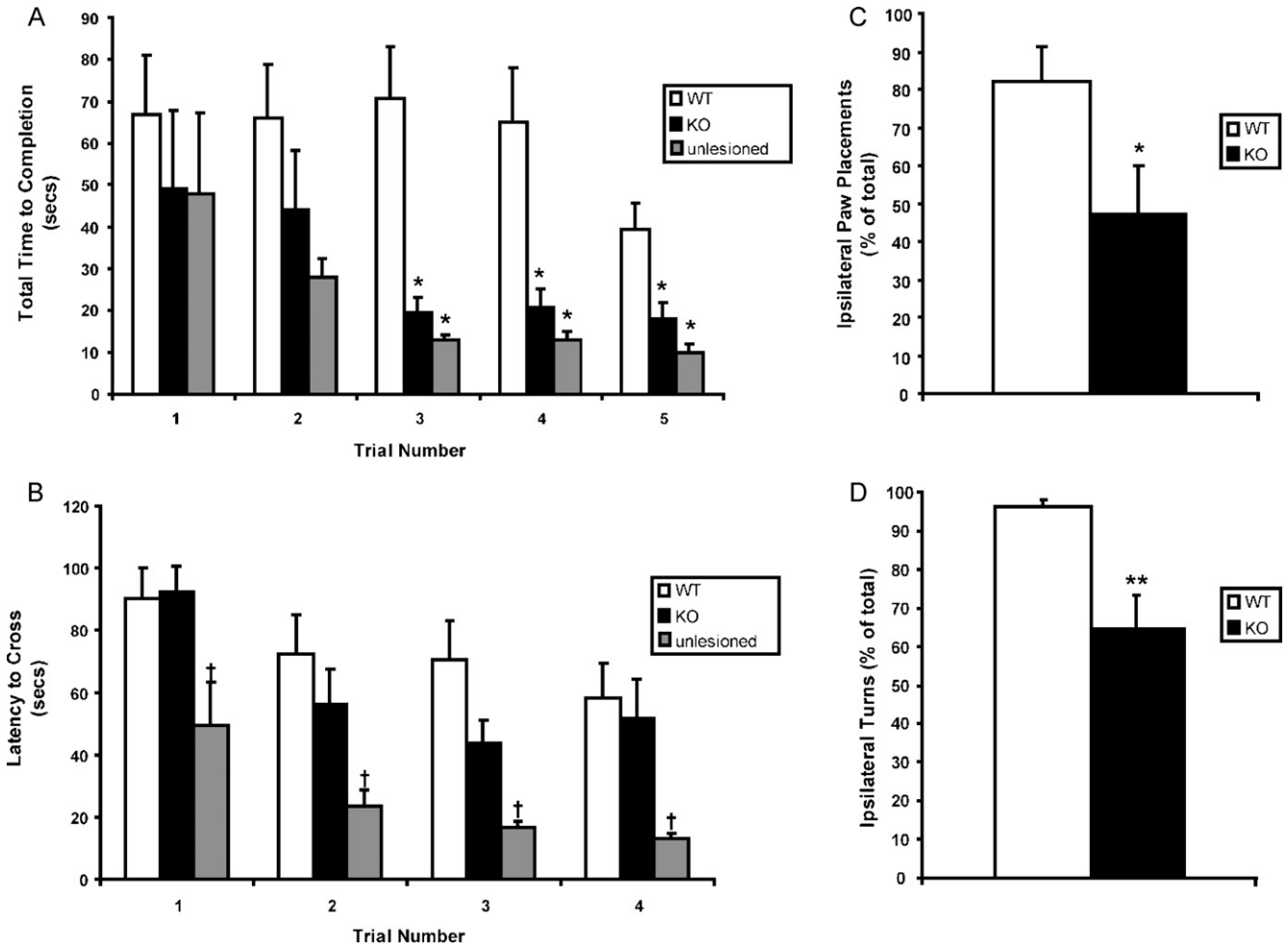


**Fig. 1.** Behavioral analyses of motor impairment and asymmetry following unilateral 6-OHDA lesions of the striatum in WT and mGluR5 KO mice. Values represent the mean (±SEM) for tests of motor impairment: vertical pole descent (A) and beam traversal task (B); and motor asymmetry: cylinder test (C) and amphetamine-induced rotation test (D) in lesioned WT/KO and unlesioned control mice. *P* values denoting different from lesioned WT mice: (†) ≤0.05, (*) ≤0.05, (**) ≤0.005.

sphere to the unlesioned right hemisphere within each animal, each mouse served as its own control. The results from biochemical analyses of striatal tissue indicated substantial reductions in residual DA and DOPAC content in the 6-OHDA lesioned hemisphere when compared to the unlesioned hemisphere in WT (DA: $t_9 = -8.73$, $P < 0.0001$; DOPAC: $t_9 = -7.48$, $P < 0.0001$) and KO (DA: $t_7 = -7.26$, $P < 0.0002$; DOPAC: $t_7 = -5.85$, $P < 0.0001$) mice (Fig. 2A). A higher baseline DA levels (intact side) was observed in the striata of KO mice compared to wild type, different with that in MPTP model of parkinsonism [4], which might be due to compensatory mechanism specific to 6-OHDA model. Though evidence of dopaminergic neuron denervation (DA loss) was exhibited in mice of both genotypes, lesioned WT mice had a substantially larger percent DA (and DOPAC) depletion than lesioned KO mice, 92% (and 80%) vs. 64% (and 52%) respectively, suggesting attenuated DA depletion and a possible neuroprotective phenotype in mGluR5 KO mice (Fig. 2B).

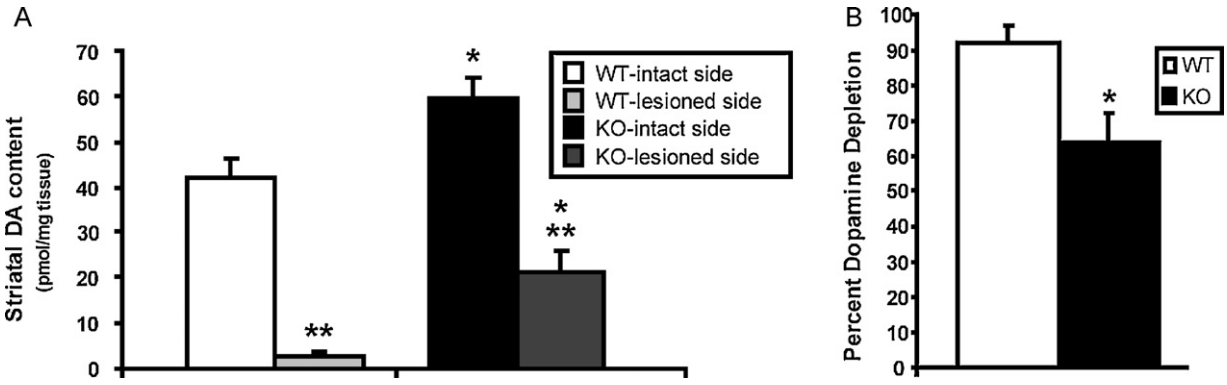


**Fig. 2.** Neurochemical comparison of striatal dopamine content in 6-OHDA lesioned vs. intact striata of WT and mGluR5 KO mice, showing attenuated dopamine depletion in mGluR5 KO. Values represent the mean (±SEM). *P* values denoting different from corresponding WT value: (*) ≤0.01; *P* values denoting different from intact side: (**) ≤0.0005.

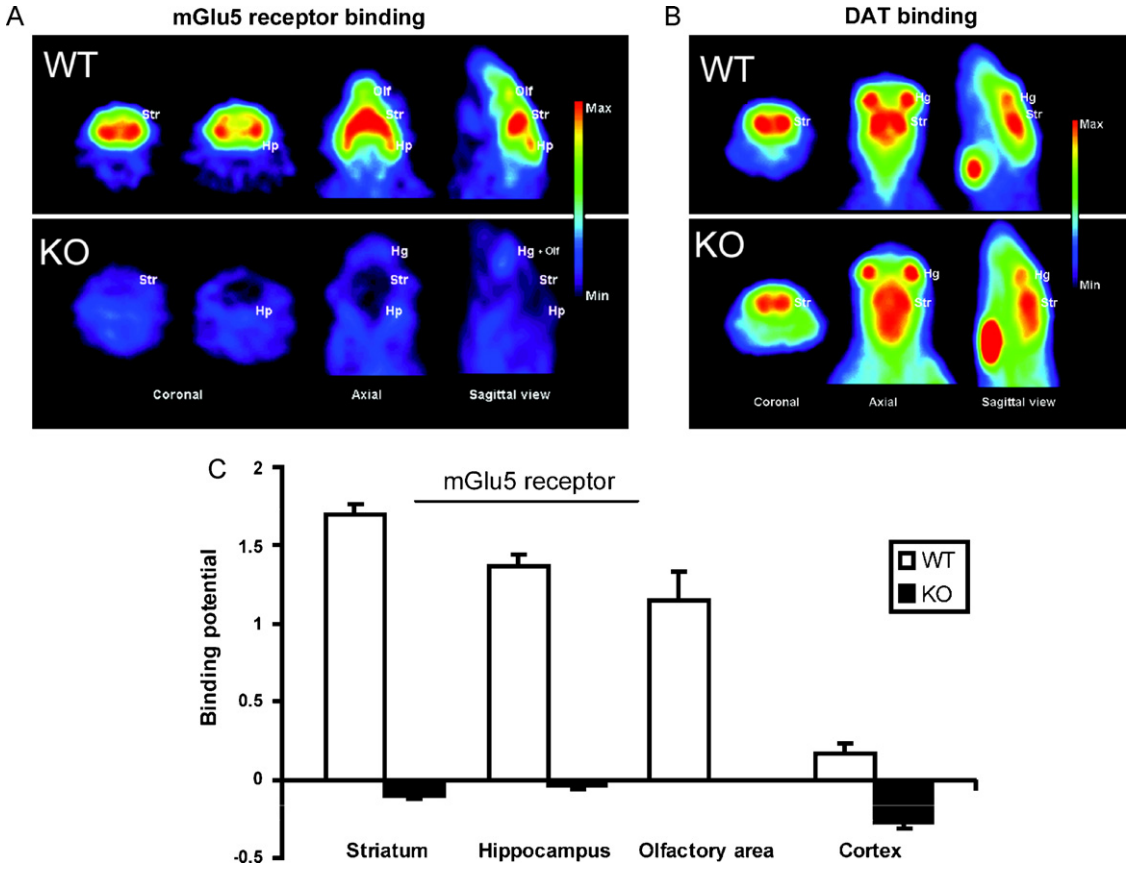


**Fig. 3.** Distribution of (A and C) mGluR5 receptor ligand [$^{18}$F]FPEB binding and (B) dopamine transporter (DAT) ligand [$^{11}$C]CFT binding in WT and mGluR5 KO mouse brain. Accumulation of [$^{18}$F]FPEB is prominent in the striatum (Str), hippocampus (Hp), Harderian glands (Hg) and olfactory area (Olf) in WT mouse brain sections (A, upper row), but absent in those of mGluR5 KO mice (A, lower row). Similar high accumulation of [$^{11}$C]CFT in the striatum of WT and KO (B) suggests that the CNS dopaminergic projection pathways are intact in the absence of the mGluR5.

As verification of receptor function, PET imaging of a separate group of unlesioned WT and mGluR5 KO mice revealed that the accumulation of [$^{18}$F]FPEB, an mGluR5 receptor ligand, is prominent in the striatum, hippocampus and olfactory bulb in WT mouse brain sections, but absent in those of mGluR5 KO mice (Fig. 3A and C). Similar high accumulation of [$^{11}$C]CFT, a selective DAT ligand, is observed in the striatum of a WT and an mGluR5 KO mouse, suggesting that the CNS dopaminergic pathways are intact in the absence of the mGluR5 receptor (Fig. 3B). The binding potentials (mean ± SEM) of [$^{18}$F]FPEB were calculated by a graphical analyzing method using the cerebellum as reference tissue (Fig. 3C).

The neurochemical and behavioral findings of the present study indicate that mGluR5 receptors play a substantial facilitative role in the dopaminergic neuron dysfunction of the 6-OHDA model of PD. Our demonstration of reduced DA loss in mGluR5 KO mice in this progressive model of nigrostriatal toxicity complements previous findings of reduced dopaminergic neuron loss in SNpc in MPTP-treated mGluR5 KO mice [4]. The attenuated 6-OHDA-induced loss of striatal DA likely reflects attenuated nigrostriatal neuron degeneration *per se*. Protection against dopaminergic neuron loss in the SNpc of MPTP-treated mGluR5 KO mice closely correlated with protection against striatal DA depletion in these mice [4]. Similarly, mGluR5 antagonist treatment attenuated both the losses of nigral dopaminergic neurons and striatal DA induced by 6-OHDA [38]. However, the ability of mGluR5 antagonists to confer protection in neuronal cultures derived from mGluR5 KO as well as WT mice [19] highlights the limitation of non-specificity with pharmacological studies, and the utility of complementary KO studies in understanding receptor function. Thus these pharmacological and KO data together comprise a consistent body of preclinical evidence for mGluR5 receptor involvement in the pathophysiology of PD.

How the receptor contributes to dopaminergic neuron degeneration is uncertain. However because the mGluR5 receptor is expressed on SNpc dopaminergic neurons themselves [16,35], it may facilitate their degeneration directly (for review see [22]), for example by mediating the excitotoxic influence of glutamatergic innervation from the subthalamic nucleus [2,7].

Similarly, our behavioral findings support a neuroprotective phenotype of mGluR5 KO mice, which performed better than WT mice in the pole and motor asymmetry tests. By contrast we found no improvement of beam walk performance, a test for motor balance and coordination, in these mice. The difference may be due to a greater performance incentive in the pole test (descending to the security of pole's home base) compared to that of our modified version of the commonly used beam walk task, (e.g. review by [10]), in which there was no home platform at the end of the beam to serve as a motivational cue. It also remains unclear whether the improved motor abnormalities observed in 6-OHDA-lesioned mGluR5 KO mice reflect a true neuroprotective phenotype (as suggested by our and others' [4] characterization of these mice in toxin models of PD), or an antiparkinsonian 'symptomatic' effect of normalized basal ganglia activity [9,18] as shown with mGluR5 antagonism in a similar 6-OHDA model [1], or both.

In conclusion, our data support roles for mGluR5 in neurodegeneration and for its blockade in neuroprotection. These findings strengthen the rationale for mGluR5 antagonism as a potential strategy to slow the underlying neurodegeneration as well as the motor dysfunction in PD.

Appendix V



## Acknowledgements

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Appendix W

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# NEUROPROTECTION BY CAFFEINE: TIME COURSE AND ROLE OF ITS METABOLITES IN THE MPTP MODEL OF PARKINSON DISEASE

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**Abstract—Epidemiological studies have raised the possibility of caffeine serving as a neuroprotective agent in Parkinson's disease (PD). This possibility has gained support from findings that dopaminergic neuron toxicity induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or other neurotoxins is attenuated by co-administration of caffeine in mice. Here we examined the time window of caffeine's neuroprotection as well as the effects of caffeine's metabolites (theophylline and paraxanthine) in the MPTP mouse model of PD. In the first experiment, caffeine pre-treatment (30 mg/kg ip) significantly attenuated MPTP-induced striatal dopamine depletion when it was given 10 min, 30 min, 1 h, or 2 h but not 6 h before MPTP (40 mg/kg ip) treatment. Meanwhile, caffeine post-treatment also significantly attenuated striatal dopamine loss when it was given 10 min, 30 min, 1 h or 2 h but not 4 h, 8 h or 24 h after MPTP injection. In the second experiment, both theophylline (10 or 20 mg/kg) and paraxanthine (10 or 30 mg/kg) administration (10 min before MPTP) significantly attenuated MPTP-induced dopamine depletion in mice, as did caffeine (10 mg/kg) treatment. Thus the metabolites of caffeine also provide neuroprotective effects in this mouse model of PD. The data suggest that if caffeine protects against putative toxin-induced dopaminergic neuron injury in humans, then precise temporal pairing between caffeine and toxin exposures may not be critical because the duration of neuroprotection by caffeine may be extended by protective effects of its major metabolites. © 2010 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: dopamine, adenosine $A_{2A}$ receptor, theophylline, paraxanthine, striatum, neurotoxicity.**

Caffeine is the most widely used psychoactive substance in the world (Fredholm et al., 1999). Multiple epidemiological studies (both prospective and retrospective) have strongly linked caffeine intake to a reduced risk of developing Parkinson's disease (PD, Benedetti et al., 2000; Ross et al., 2000; Ascherio et al., 2001), raising the possibility of neuroprotection by caffeine. This hypothesis has been strengthened by preclinical demonstrations of neuroprotection by caffeine in a variety of neurotoxin models of PD. Caffeine attenuated neurochemical and anatomical dopaminergic lesions induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Chen et al., 2001, 2008; Xu et al., 2002, 2006a; Singh et al., 2009), 6-hydroxydopamine (Joghataie et al., 2004; Aguiar et al., 2006), or the pesticide combination of paraquat plus maneb (Kachroo et al., 2007). Moreover in contrast to the motor stimulant properties of caffeine, which show tolerance upon repeated exposure, the neuroprotective effect of caffeine was not reduced after repeated administration (Xu et al., 2002).

In the MPTP model of PD the neuroprotective effect of caffeine has been assessed by effectively co-administering it together with the toxicant, with caffeine preceding MPTP by 10 min. In humans, however, caffeine intake typically occurs once or a couple of times a day in a periodic fashion, and thus it may not necessarily pair together with the potential toxin exposure. Here we investigate caffeine pharmacokinetics and the temporal requirements for pairing caffeine and neurotoxin in mice in an effort to better understand the relationship between caffeine intake and reduced risk of PD. We employ a single-dose toxin regimen (MPTP·HCl 40 mg/kg i.p.) because it affords simplicity in assessing the temporal features of protectant-toxicant relationships, and because it is the most common toxicant paradigm used in earlier studies of caffeine in PD models (Chen et al., 2001; Xu et al., 2002, 2006a). Despite its limitations (e.g., the lack of Lewy body-like inclusions or reliable behavioral deficits characteristic of PD) acute MPTP intoxication in mice remains one of the best-characterized animal models of PD and faithfully recapitulates core neurochemical and anatomical features of PD (Dawson et al., 2002).

To pursue the pharmacodynamics of neuroprotection by caffeine we considered the major routes of caffeine metabolism. In human adults caffeine is virtually completely metabolized, with less than 2% of the ingested compound being recoverable in urine unchanged (Arnaud, 1987; Somani and Gupta, 1988). In humans caffeine (i.e., 1,3,7-trimethylxanthine) is demethylated to its dimethyl metabolic intermediates, with over 80% of orally administered caffeine metabolized to paraxanthine (1,7-dimethylxanthine), and about 16% is converted to theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) (Lelo et al., 1986; Benowitz et al., 1995). In rodents, paraxanthine is the major metabolite, but levels of theophylline are also high (Bonati et al., 1984–1985; Fredholm et al., 1999). Paraxanthine can contribute to the pharmacological action of caffeine (Benowitz et al., 1995), especially during long-term caffeine consumption at higher doses

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*Abbreviations:* MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease.

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when there is accumulation of paraxanthine in plasma due to its saturable metabolism (Denaro et al., 1990). These metabolites of caffeine share many of the pharmacological actions of caffeine (Denaro et al., 1990). Thus they should be taken into account when considering the biological actions of caffeine. Therefore, in the current study, we examined the effects of caffeine's metabolites (theophylline and paraxanthine) as well as its time window of action in the MPTP model of PD.

## EXPERIMENTAL PROCEDURES

### Animals and drug treatments

Male C57BL/6 mice (~23–27 g) from Charles River Laboratories (Wilmington, MA, USA) were housed five in each cage in temperature- and humidity-controlled rooms with a 12-h dark: light cycle and had free access to food and water. In the first experiment, mice received saline or caffeine (30 mg/kg) 10 min, 30 min, 1 h, 2 h or 6 h before (Experiment 1A), or 10 min, 30 min, 1 h, 2 h, 4 h, 8 h or 24 h after (Experiment 1B) MPTP (40 mg/kg, $n$=7–20; note all doses for MPTP refer to that of its chloride salt, MPTP·HCl; Sigma-Aldrich, St. Louis, MO, USA) or saline ($n$=3–6) treatment. In the second experiment, caffeine (10 mg/kg), theophylline (10 or 20 mg/kg), paraxanthine (10 or 30 mg/kg) or saline was administered 10 min before MPTP (40 mg/kg, $n$=7–20) or saline ($n$=3–7) treatment. In the third experiment, mice were treated with caffeine (10 or 30 mg/kg) and killed at 5, 10, 20, 30, 60, 120, 360 min or 24 h ($n$=6 for each time point) after injection. All injections were administered i.p. in a volume of 0.1 ml/10 g of body weight. All experiments were conducted in accordance with Massachusetts General Hospital and NIH Guidelines on the ethical use of animals. AQ: 1

### Measurement of dopamine

In the first two experiments, 1 week after MPTP treatment, mice were killed by rapid cervical dislocation. The striatum was dissected out from the right cerebral hemisphere, frozen on dry ice and stored at −80 °C until use. Each striatum was weighed, homogenized with 150 mM phosphoric acid and 0.2 mM EDTA and centrifuged at 12,000×$g$ for 15 min at 4 °C. Supernatants were analyzed for dopamine content using standard reverse-phase HPLC with electrochemical detection. Biogenic amines were separated on a C-18, 5-μm sphere column. The mobile phase consisted of 0.1 M sodium phosphate monobasic, 0.1 mM EDTA, 0.18 mM sodium octyl sulfate, and 8% methanol in filtered distilled water. A final pH of 3.3 was obtained with the addition of concentrated phosphoric acid, and the mobile phase was filtered and degassed before use.

### Measurement of caffeine and its metabolites

In the third experiment, mice were killed at different time points by rapid cervical dislocation. Trunk blood was collected and centrifuged immediately at 12,000×$g$ for 30 min at 4 °C. The right cerebral hemisphere was dissected out, frozen on dry ice, and stored at −80 °C until use. Brain tissue was homogenized in 0.1 M monobasic sodium phosphate with a volume 10 times that of tissue weight (i.e., 10 μl/mg tissue assuming a tissue density of 1 μl/mg) and centrifuged at 12,000×$g$ for 15 min at 4 °C. Serum and supernatant of brain homogenates were analyzed using liquid chromatography/mass spectrometry for determination of caffeine and its three dimethyl xanthine metabolites: paraxanthine, theophylline, and theobromine. The lower limit of quantitation was 0.030 μg/ml. The results of caffeine and metabolites concentrations in brain were calculated (i.e., μg/ml homogenate×10 ml homogenate/g tissue) and presented as μg/g tissue. The analysis of caffeine and its metabolites was performed by Drs. R. L. Foltz and D. Andrenyak (Center for Human Toxicology, University of Utah, Salt Lake City, UT, USA) as a service of the National Institute of Drug Abuse (NIDA Drug Supply & Analytical Services; Dr. H.H. Singh, program administrator).

### Statistics

The dopamine content data from MPTP-treated mice were analyzed with one-way ANOVA followed by Fisher's LSD tests. AQ: 2

## RESULTS

In the first experiment, we sought to determine the therapeutic window through which pre- or post-treatment with caffeine confers neuroprotection against MPTP-induced dopaminergic toxicity in mice (Fig. 1). Caffeine (30 mg/kg i.p.) significantly attenuated MPTP-induced dopamine depletion not only when it was effectively co-administered with (10 min before) MPTP ($P$<0.001) as demonstrated previously (Chen et al., 2001), but also when it was given 30 min ($P$<0.001), 1 h ($P$<0.001) or 2 h ($P$<0.01) before MPTP treatment. However when caffeine administration preceded that of MPTP by 6 h, then any attenuation of dopamine loss did not reach statistical significance (Fig. 1A). Similarly, when toxin exposure preceded caffeine treatment, striatal dopamine depletion was significantly reduced if caffeine was given 10 min ($P$<0.001), 30 min ($P$<0.001), 1 h ($P$<0.001) or 2 h ($P$<0.01) after MPTP treatment. However, MPTP-induced striatal dopamine depletion was not appreciably altered when caffeine was given 4, 8 or 24 h after MPTP (Fig. 1B). F1

To further characterize the mechanism of caffeine's neuroprotection in the MPTP mouse model of PD, in the second experiment we investigated the effects of caffeine metabolites theophylline and paraxanthine upon MPTP-induced striatal dopaminergic toxicity. Both theophylline (10 mg/kg, $P$<0.05; 20 mg/kg, $P$<0.05) and paraxanthine (10 mg/kg, $P$<0.05; 30 mg/kg, $P$<0.01) pre-treatments (10 min before MPTP) significantly attenuated MPTP-induced dopamine depletion in mice, as observed with the caffeine (10 mg/kg, $P$<0.01) pre-treatment (Fig. 2). F2

We also measured the concentrations of caffeine and its metabolites in serum and brain at different time points after injection (Fig. 3). Caffeine concentrations in serum were higher than those in the brain at all time points. The brain levels of caffeine were about 54%–65% of blood levels. Serum and brain concentrations of caffeine reached a peak at 10 min after injection (30 mg/kg ip). At 60 min after injection, the concentrations were about 50% of maximal values. Six hours after injection, caffeine levels were close to zero in both serum and brain. Caffeine could not be detected in either serum or brain 24 h after injection. Meanwhile, the concentrations of caffeine's metabolites (theophylline, paraxanthine, and theobromine) increased steadily after injection (from 5 to 120 min), though they were much lower than those of caffeine for at least 2 h after injection. The levels of metabolites were close to zero 6 h and could not be detected 24 h after injection. Paraxanthine was the major metabolite and its levels F3



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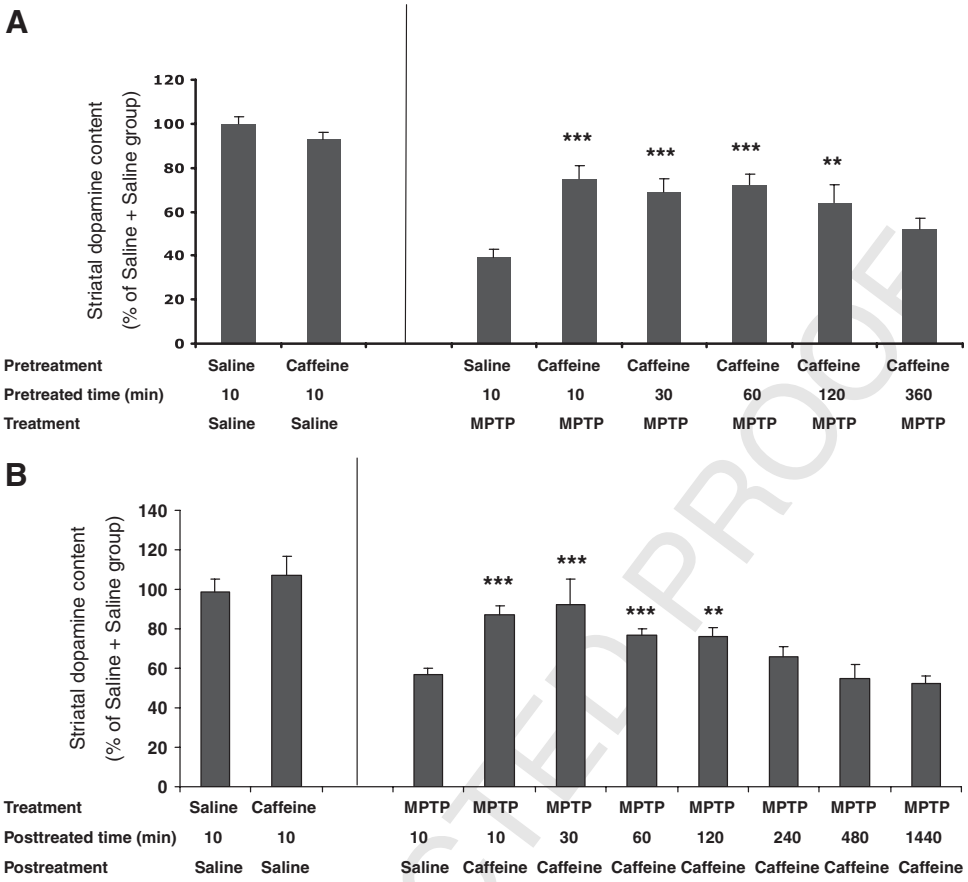


**Fig. 1.** (A) Caffeine pre-treatment significantly attenuates MPTP-induced dopaminergic toxicity. C57Bl/6 male mice received saline or caffeine (30 mg/kg ip) at different time points before MPTP (40 mg/kg, $n=7–9$) or saline ($n=3–4$) ip treatment. Striatal dopamine content was determined 1 wk later. Bars represent striatal dopamine levels (mean±SEM) calculated as percentage of their control (i.e., saline 10 min before saline treatment group, in which dopamine content is 65±2.1 pmol/mg tissue). Data were analyzed with one-way ANOVA followed by Fisher's LSD test. ** $P<0.01$, *** $P<0.001$ when compared with saline 10 min before MPTP treatment group. (B) Caffeine post-treatment significantly attenuates MPTP-induced dopaminergic toxicity. C57Bl/6 male mice received saline or caffeine (30 mg/kg ip) at different time points after MPTP (40 mg/kg, $n=7–20$) or saline ($n=5–6$) ip treatment. Striatal dopamine content was determined 1 week after MPTP. Bars represent striatal dopamine levels (mean±SEM) calculated as percentage of their control (i.e., saline 10 min post saline treatment group in which dopamine content is 42±4.8 pmol/mg tissue). Data were analyzed with one-way ANOVA followed by Fisher's LSD test. ** $P<0.01$, *** $P<0.001$ when compared with saline 10 min post MPTP treatment group.

were higher than those of theophylline or theobromine at all time points. When administered at a 3-fold lower dose (10 mg/kg), caffeine showed a similar pharmacokinetic pattern with a proportionally smaller peak also seen 10 min after administration.

## DISCUSSION

The results of current study demonstrated that caffeine's neuroprotective effect in the MPTP mouse model of PD can be achieved when administered either preceding or following toxin exposure for up to 2 h. Moreover, caffeine's neuroprotective effect was mimicked by comparable doses of its dimethyl metabolites theophylline and paraxanthine.

Interestingly, like caffeine, two of its dimethylxanthine metabolites displayed similar neuroprotective properties in this mouse model of PD. Both paraxanthine and theophylline, two major metabolites of caffeine in humans as well as rodents, attenuated the loss of striatal dopamine induced by acute MPTP intoxication. These metabolites share many features of caffeine's actions including those affecting CNS. For example, theophylline and paraxanthine (using doses similar to those applied in this study) enhance motor (e.g., rotational or locomotor) behaviors in rodents as does caffeine (Watanabe et al., 1982; Logan et al., 1986). Paraxanthine and theophylline also share pharmacological properties with caffeine as all three methylxanthines are adenosine receptor antagonists, phosphodiesterase inhibitors and capable of releasing intracellular calcium stores. However, relatively high concentrations of caffeine or its metabolites are required to exert their effects on phosphodiesterase and calcium release (Fredholm et al., 1999; Guerreiro et al., 2008). By contrast, lower dosages and concentrations are sufficient to achieve blockade of adenosine receptors. For example, serum caffeine concentrations produced by the ingestion of a single cup of caffeinated coffee are sufficient to significantly displace endogenous adenosine from $A_1$ and $A_{2A}$ receptors, whereas near toxic exposures to caffeine are required to

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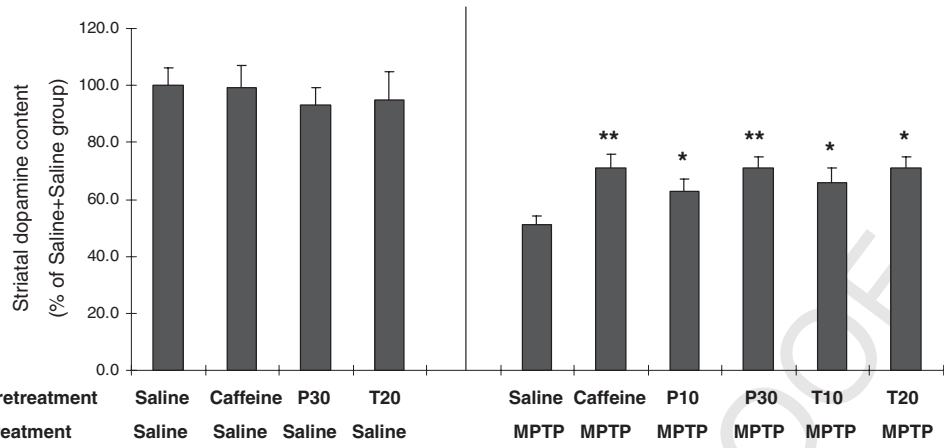


**Fig. 2.** Caffeine metabolites significantly attenuate MPTP-induced dopaminergic toxicity. C57Bl/6 male mice received ip injection of caffeine (10 mg/kg), theophylline (10 or 20 mg/kg), paraxanthine (10 or 30 mg/kg) or saline 10 min before MPTP (40 mg/kg, $n$=7–20) or saline ($n$=3–7) ip treatment. Striatal dopamine content was determined 1 week later. Bars represent striatal dopamine levels (mean±SEM) calculated as percentage of their control (i.e., saline 10 min before saline treatment group in which dopamine content is 58±3.5 pmol/mg tissue). P10 or P30, paraxanthine 10 or 30 mg/kg, respectively; T10 or T20, theophylline 10 or 20 mg/kg, respectively. Data were analyzed with one-way ANOVA followed by Fisher's LSD test. * $P<0.05$, *** $P<0.01$ when compared with saline 10 min before MPTP treatment group.

produce meaningfully inhibit phosphodiesterase activity. Furthermore, the behavioral stimulant potencies of caffeine and its metabolites correlate with their affinities for occupation of adenosine receptors (Kaplan et al., 1997). Theophylline is more potent than caffeine as an inhibitor of both adenosine $A_1$ and $A_{2A}$ receptors, and paraxanthine is also at least as potent as caffeine (Mally and Stone, 1994). In the current experiments, the brain concentrations of caffeine (as shown in Fig. 3B) ranged from ~32 to 110 $\mu$M after 30 mg/kg ip, and ~26 to 44 $\mu$M after 10 mg/kg ip. At these levels, caffeine mainly blocks adenosine $A_1$ and $A_{2A}$ receptors, although other mechanisms cannot be excluded (Fredholm et al., 1999). We have demonstrated previously that the neuroprotective effect of caffeine probably involves its antagonism at the $A_{2A}$ adenosine receptor. For example, selective adenosine $A_{2A}$, but not $A_1$, antagonists (e.g., SCH58261 and KW6002) provided similar protection against MPTP toxicity in mice as those treated with caffeine (Chen et al., 2001). Moreover, caffeine's neuroprotective effect was lost in $A_{2A}$ knockout mice that lack functional $A_{2A}$ receptors (Xu et al., 2006b). Thus, the neuroprotective effects of theophylline and paraxanthine may similarly rely on their actions as $A_{2A}$ receptor antagonists. Although paraxanthine has been found to protect dopaminergic neurons in culture by an $A_{2A}$ receptor-independent mechanism, the concentrations employed were greater than those that bind adenosine receptors or that are achieved *in vivo* (Fredholm et al., 1999; Guerreiro et al., 2008). These findings are consistent with the hypothesis that caffeine or other methylxanthines confer protection on dopaminergic neurons *in vivo* by $A_{2A}$ receptors that are expressed on non-dopaminergic neurons (Xu et al., 2005).

The mechanism by which caffeine and in turn blockade of adenosine $A_{2A}$ receptors protect against MPTP-induced dopaminergic toxicity remains uncertain, although various hypotheses have been suggested (Chen et al., 2008; Yu et al., 2008; Carta et al., 2009). MPTP is metabolized to $MPDP^+$ and then oxidized to active metabolite $MPP^+$. Previously, we have shown that caffeine does not change MPTP's entry into the brain or its metabolism to $MPDP^+$ and $MPP^+$ in the C57Bl/6 mice (Chen et al., 2001, 2002). $MPP^+$ level peaks at 90 min after single injection (Giovanni et al., 1991; Chen et al., 2002). Therefore, our current demonstration that caffeine produces its full protective effect even when administered 2 h after MPTP injection further substantiates that caffeine's action occurs downstream of MPTP entry into the CNS and of its conversion to the active dopaminergic neuron toxin $MPP^+$.

The pharmacokinetic profile of caffeine and its metabolites corresponded closely with the functional time course of neuroprotection by caffeine in the present study. Six hours after caffeine injection, caffeine cannot be detected, and only very low levels of paraxanthine or theobromine can be detected in the blood and brain. Similarly, we did not see caffeine's protection when it was given 6 h before MPTP. Thus the physical presence of caffeine or related methylxanthine and their direct action (for example, blockade of $A_{2A}$ receptors) might be required in order for caffeine to disrupt a critical early pathophysiological step in the injury of dopaminergic neurons.

We found that caffeine's half-life was about 60 min in both blood and brain in C57Bl/6 male mice agreeing with that found in CD1 and CD-COBS mice (Bonati et al., 1984–1985; Kaplan et al., 1989, 1990), accounting in part for the relatively narrow time window of protection by caffeine. In addition, although paraxanthine was found to be protective and to be the major dimethylxanthine metabolite of caffeine in C57Bl/6 mice, its levels (as well as the levels of the other two metabolites) are much lower than those of caffeine at all time points studied. Similar differences between levels of caffeine and its metabolites were found in CD-COBS (Bonati et al., 1984–1985) and DBA/2J (Kuzmin et al., 2000) mice. However, caffeine's metabolism varies considerably among different species of animals and hu-



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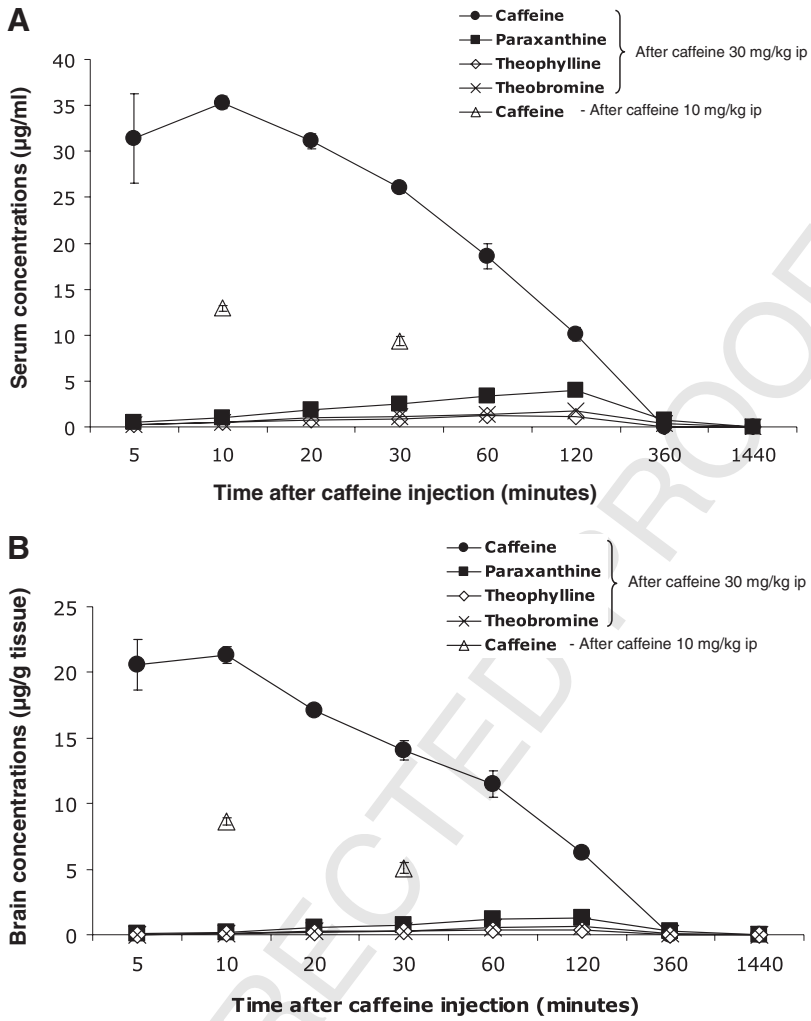


**Fig. 3.** Serum (A) and brain (B) levels of caffeine and its metabolites after caffeine injection. C57bl/6 male mice were treated with caffeine (10 or 30 mg/kg ip) and killed at 5, 10, 20, 30, 60, 120, 360 min or 24 h ($n$=6 for each time point) after injection. Serum and brain concentrations of caffeine and its metabolites (paraxanthine, theophylline, and theobromine) were determined.

mans. For example, in humans, caffeine's half life was found to be around 2.5–4.5 h (Bonati et al., 1984–1985; Arnaud, 1987) instead of ~1 h in rodents. Moreover, paraxanthine levels were found to be more than one-third of caffeine levels in humans while paraxanthine level was a much smaller fraction (~one-tenth to one-fifth) of that of caffeine in rodents (Bonati et al., 1984–1985 and current study). Thus the demonstration of neuroprotection by paraxanthine and theophylline may contribute only modestly to caffeine's neuroprotective effect in C57Bl/6 mice employed here. Of practical significance, the relative brevity of the 4 h period straddling MPTP exposure in which protection was demonstrated suggests that more continuous caffeine exposure may be required to test its protective potential in chronic toxin or genetic mouse models of PD.

In humans however, the relatively prolonged half-life of caffeine, which results in elevated levels of caffeine and paraxanthine after bolus intake (Bruce et al., 1986), together with the newly identified neuroprotective potential of its primary metabolite paraxanthine, raise the possibility that any true protective effect of individual caffeine ingestions could be relatively long-lasting. Epidemiological studies linking higher rates of caffeine consumption to a reduced risk of PD showed the reduced risk was greatest for those consuming multiple cups of coffee per day (Benedetti et al., 2000; Ross et al., 2000; Ascherio et al., 2001), in whom significant blockade of adenosine receptors may have been achieved by dimethylxanthine metabolites as much as by caffeine throughout much of the day. The current findings demonstrate that caffeine must be paired with the dopaminergic neuron toxin MPTP (i.e., with co-administration or a stagger of no more than a few hours) in order for its neuroprotective effect to be realized. By contrast in humans, if a neuroprotective effect of caffeine were in fact the basis of the reduced risk amongst caffeine consumers, then close pairing between caffeine and episodic exposures to putative environmental neurotoxins contributing to PD may not be necessary. This temporal relationship between caffeine and toxin exposures in the MPTP model and its extrapolation to humans has



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implications for possible neuroprotection trials of caffeine in PD, which have been suggested based on available epidemiological and laboratory data (Richardson et al., 1997; Kanda et al., 1998; Grondin et al., 1999; Ravina et al., 2003) despite a lack of demonstrated association between caffeine use and clinical progression in PD (Schwarzschild et al., 2003; Simon et al., 2008) For example, a regimen of twice or thrice daily caffeine dosing could produce effectively continuous blockade of adenosine receptors and allow for a reliable pharmacological design for such a trial.

Clinical relevance of the present findings also arises given the routine long-term use of theophylline to treat asthma. A protective effect of theophylline in a mouse model of PD raises the possibility that asthmatics chronically treated with theophylline may be at reduced risk for developing PD. Although theophylline use is routine its prevalence (e.g., relative to caffeine exposure) is modest and unlikely to be informative in prospective epidemiological studies of PD. Nevertheless, in light of its established safety record for chronic pharmacotherapy in clinical practice our findings suggest that it may also be considered as a candidate disease-modifying treatment for PD. Indeed based on its known adenosine receptor antagonism and preclinical benefits for motor control, theophylline has been tested in small-scale clinical trials for patients with PD resulting in both positive (Mally and Stone, 1994) and negative (Magnussen et al., 1977; Kulisevsky et al., 2002) results. However, like selective $A_{2A}$ receptor antagonists in clinical trials for PD (currently developed by multiple pharmaceutical companies such as Kyowa-Kirin, Schering-Plough, Biogen-Idec, etc.), neither theophylline nor caffeine has yet been investigated in a trial designed to assess long-term outcomes in PD. Our findings support the practicality of considering natural methylxanthines with adenosine antagonist properties as candidate neuroprotectants in PD.

AQ: 4

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AQ: 5

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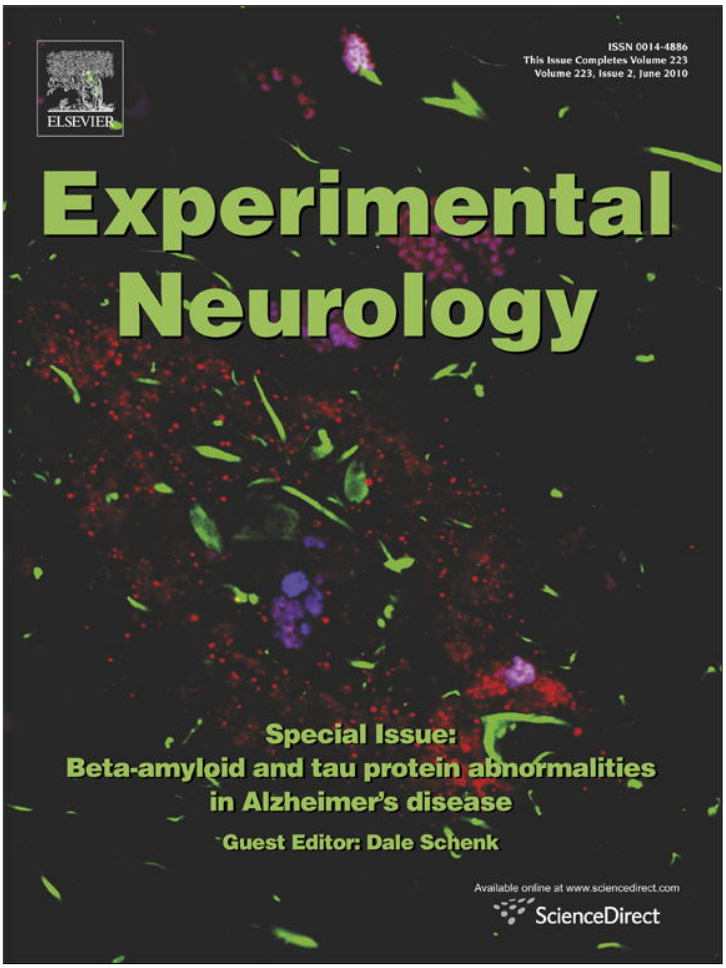
Appendix X



Appendix X

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Brief Communication

# Caffeine protects against combined paraquat and maneb-induced dopaminergic neuron degeneration

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ABSTRACT

Environmental exposures suspected of contributing to the pathophysiology of Parkinson's disease (PD) include potentially neurotoxic pesticides, which have been linked to an increased risk of PD. Conversely, possible protective factors such as the adenosine antagonist caffeine have been linked to a reduced risk of the disease. Here we assessed whether caffeine alters dopaminergic neuron loss induced by exposure to environmentally relevant pesticides (paraquat and maneb) over 8 weeks. The number of nigral neurons positive for tyrosine hydroxylase immunoreactivity (TH+) was assessed using stereological methods and found to be significantly reduced (to 60% of control) by combined pesticide treatment. Caffeine at 20 mg/kg significantly reduced TH+ neuron loss (to 85% of the respective control). The results demonstrate the neuroprotective potential of caffeine in a chronic pesticide exposure model of model of PD.



Parkinson's disease (PD) is a chronic neurodegenerative condition with cardinal clinical features of rest tremor, rigidity, and bradykinesia attributed to an underlying neurodegeneration of dopaminergic neurons of the substantia nigra. Evidence suggests both genetic and environmental risk factors for PD (Warner and Schapira, 2003; Mizuno et al., 1999). Nongenetic factors appear to be prominent in the majority of sporadic PD patients, in whom typical symptoms develop after age 50. Twin studies (Tanner et al., 1987; Wirdefeldt et al., 2004) have generally found indistinguishable concordance rates for PD diagnosed after age 50 between monozygotic and dizygotic twins, supporting a prominent role for environmental risk factors.

Amongst environmental factors, exposure to pesticides has been linked to an increase in the risk of PD (Chade et al., 2006; Tanner et al., 2009; Costello et al., 2009). In addition to case–control studies, several prospective epidemiological investigations have supported an elevated risk of developing PD among those exposed to pesticides. In Hawaiian men of Japanese ancestry, the relative risk of developing PD tended to be increased (though did not reach statistical significance) for those individuals exposed to pesticides (Petrovitch et al., 2002). In an investigation of elderly subjects residing in France, a significant increase in the relative risk of PD among men, but not women, was associated with occupational pesticide exposure (Baldi et al., 2003).

Recently, a large prospective epidemiological study (Ascherio et al., 2006) of both men and women in the United States found that current or regular past exposure to pesticides was associated with an increased risk for developing PD. Pesticide use was associated with a 70% increased risk of developing PD among men and women whose pesticide exposure history was reported before diagnosis. Although this study addressed the concern of recall bias that limited interpretation of prior studies, it did not identify specific pesticides as potential environmental risk factors for PD. While most studies have shown a positive association between pesticide exposure and PD, no specific single agent has been implicated consistently. The herbicide paraquat (PQ) has emerged as a putative human risk factor based on epidemiologic and occupational exposure data (Hertzman et al., 1990; Liou et al., 1997; Tanner et al., 2009) as well as experimental cell (McCarthy et al., 2004) and rodent data (Brooks et al., 1999; McCormack et al., 2002; Prasad et al., 2007).

Exposures to pesticides such as PQ are likely to occur in combination with other pesticides because single agents are often applied in overlapping geographical areas (Thiruchelvam et al., 2000a). For example, diethyldithiocarbamates, of which maneb (MB) is a member, are heavily used alongside PQ in certain parts of the United States (United States Geographic Service, 1998). Occupational exposure to MB has been anecdotally linked to cases of parkinsonism in humans (Ferraz et al., 1988; Meco et al., 1994).

Thiruchelvam et al. (2000a,b) established a dual-pesticide model of environmental parkinsonism in mice based on repeated systemic

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exposure to a combination of PQ and MB. Coadministration of these toxins was shown to produce selective loss of nigrostriatal dopaminergic neurons and loss of dopamine in the striatum. In addition to its potential environmental significance, this 'dual-pesticide' mouse model entails exposure to PQ and MB over months and thus parallels the long-term exposure in humans to putative environmental neurotoxicants, further enhancing its utility in the characterization of promising neuroprotective candidates for PD.

In contrast to pesticide exposure, the consumption of coffee and other caffeinated beverages has been repeatedly linked to a *reduced* risk of developing PD (Xu et al., 2005). Caffeine itself appears to be the component that accounts for the association given that caffeinated, but not decaffeinated coffee, was found to be associated with a lower PD risk in a large prospective study (Ascherio et al., 2001).

Laboratory studies from our and other groups have complemented these epidemiologic findings to suggest that the inverse association between caffeine and PD risk may be due to a direct neuroprotective action of caffeine. We found caffeine at a dose in mice corresponding to as little as a single cup of coffee in humans could significantly attenuate the loss of striatal dopamine induced by acute exposure to the dopaminergic neuron toxin MPTP (Chen et al., 2001). Similarly, caffeine can reduce nigrostriatal neuron injury triggered by a single dose of locally administered 6-OHDA in rats (Joghataie et al., 2004; Aguiar et al., 2006). The biological plausibility of a neuroprotective action of caffeine in PD was strengthened by the demonstration that antagonists of the adenosine $A_{2A}$ (but not $A_1$) receptor mimic the effects of caffeine in these acute MPTP and 6-OHDA toxin models of PD (Chen et al., 2001; Ikeda et al., 2002; Pierri et al., 2005; Bove et al., 2005). Genetic disruption of the $A_{2A}$ receptor similarly attenuated acute neurotoxicity in the MPTP mouse model of PD (Chen et al., 2001).

These convergent epidemiologic and laboratory data have prompted the inclusion of caffeine on a short list of candidate neuroprotective agents warranting consideration for disease-modifying therapy in clinical neuroprotection trials for PD (Ravina et al., 2003). However, the recent failure of several clinical neuroprotection trials of agents that had emerged as promising candidates from the preclinical pipeline for PD has prompted a reexamination of the animal models supporting the pipeline as well as the clinical trial methodology used to test the candidates (Hung and Schwarzschild, 2007; Suchowersky et al., 2006). These concerns have encouraged the development and use of additional animal models of PD (beyond the historical acute toxin models), with particular emphasis on models that are more intuitively relevant to PD, for example, because they better mimic the pathology or time course of the disease (Fornai et al., 2005; Anderson et al., 2006). Accordingly, the current study seeks to better assess the neuroprotective potential of caffeine by testing its ability to alter the dopaminergic nigral neuron degeneration induced by chronic exposure to pesticides (Thiruchelvam et al., 2000a,b).

Two-month-old male C57BL/6NCrl mice were obtained from Charles River Laboratories; Wilmington, MA and housed under a 12:12 hr light–dark cycle. Food and water was provided *ad libitum*. All experiments were performed in accordance with Massachusetts General Hospital and NIH guidelines on the ethical use of animals, with adequate measures taken to minimize pain and discomfort. Mice were injected i.p. with saline (vehicle), caffeine 5 mg/kg or caffeine 20 mg/kg (Sigma), followed 10 minutes later by a pair of i.p. injections, either saline for both or 10 mg/kg PQ (1,1′-dimethyl-4,4′-bipyridinium) dichloride hydrate (Sigma) first and 30 mg/kg MB (manganese bisethylenedithiocarbamate) (Chem Service) second. PQ and MB were dissolved separately in saline on the day of administration. Mice were treated chronically (twice weekly for 8 weeks) in the following initial randomly assigned groups: saline control ($n=8$); PQ and MB ($n=12$), caffeine (5 mg/kg) control ($n=8$), caffeine (5 mg/kg) + (PQ and MB) ($n=12$), caffeine (20 mg/kg) control ($n=8$), and caffeine (20 mg/kg) + (PQ and MB) ($n=12$). A mortality rate of 42% in the toxin-treated groups consistent with the upper range of prior experience in this PQ and MB paradigm (M. Thiruchelvam, personal communication) occurred equally (5 of 12 mice) across these three pesticide groups and is thus unlikely to affect comparisons among them. Toxin treatment-related deaths occurred between the 2nd and 13th dose with the majority of mice expiring between the 4th and 5th dose. Mortality was low (0–25%) in the three control groups not treated with toxins, leaving final $n=8$, $n=7$, and $n=6$ for all analyses in control groups pretreated with 0, 5, and 20 mg/kg caffeine, respectively. Body weights were obtained twice a week during the course of the experiment. No differences in body weight were produced by any of the treatments, consistent with previous observations (Thiruchelvam et al., 2000a). Animals were sacrificed 1 week after the last injection.

During the experimental paradigm, horizontal locomotor activity subdivided into ambulation and fine movement, was assessed by an automated recording system (San Diego Instruments) in standard polypropylene cages (15×25 cm) placed into frames equipped with 5 infrared photocell beams (5 cm apart). Ambulation was measured as the number of sequential breaks in 2 adjacent beams, and fine motor activity (which can reflect grooming and other stereotyped activities) was measured as the number of sequential breaks in a single beam. Photobeam breaks were recorded in consecutive 10-minute periods for 3 hours. Mice were not tested on treatment days but 24–48 hours later. Motor assessments were performed at baseline (day 0), and on the 4th, 6th, and 8th week time points over the 8-week treatment period for a total of 4 sessions. No differences in motor activity (measured over the full 3-hour testing session or just the first 45 minutes or last 60-minute periods) were observed during the course of the experiment due to any of the treatments (data not shown).

One week after the last injection (week 9), mouse brains were removed and immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored at 4 °C overnight. The paraformaldehyde-fixed brains were then transferred to 30% sucrose for cryoprotection. Brains were rinsed in PBS, placed in aluminum foil, and stored at −80 °C. The fixed brains were cut on a Leica microtome into 40-μm-thick sections for immunolabelling studies. Free-floating sections were washed three times in PBS and treated with a 20:1 mixture of 3% hydrogen peroxide and Triton X-100. The sections were washed again three times in PBS before incubation in a blocking solution of 3% milk in PBS for 1 hour. After a further three washes, sections were incubated overnight at 4 ºC with a primary antibody to tyrosine hydroxylase (monoclonal anti-TH, 1:800; Sigma). The next day, the sections were washed three times before incubation with a secondary biotinylated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) at 1:200 for 1 hour followed by three washes and incubation for 1 hour in ABC solution (Vectastatin; Vector Labs), before the sections were further washed in PBS and developed in 0.3 mg/ml 3-3′-diaminobenzidine tetrachloride (DAB). The stained free-floating sections were mounted on glass slides and coverslipped. These slides were counterstained with cresyl violet acetate (9-amino-5-imino-5*H*-benzo[*a*]phenoxazine acetate salt; Sigma). Complete antibody penetration was verified to cover the full thickness of sections using confocal microscopy (Melvin and Sutherland, 2009).

Stereological assessment of neuronal loss in midbrain sections was limited to the substantia nigra pars compacta (SNpc) because Thiruchelvam et al. (2000b) had previously demonstrated that neuronal cell counts of the ventral tegmental area were unaffected in mice exposed to paraquat and maneb. After delineation of the SNpc at low magnification (10× objectives), the entire region was sampled at higher magnification (40× objectives). The total number of TH+ and TH− neurons in the SNpc were counted following specified criteria (West et al., 1993; Chan et al., 1997) using the optical dissector technique in 40-μm-thick coronal sections throughout the entire substantia nigra bilaterally. The entire volume of the SN was estimated according to the principle of Cavalieri (1966) using the Bioquant Image

Analysis System (R&M Biometrics, Nashville, TN). TH-immunoreactive neurons were counted using 75 μm × 75 μm optical dissectors at 40× power, excluding neurons in the superficial plane of section. All counts were performed by a single investigator blinded as to the treatments. The average coefficient of error (CE) from the sampling technique was 0.089 and 0.083 for the TH+ and TH− counts, respectively.

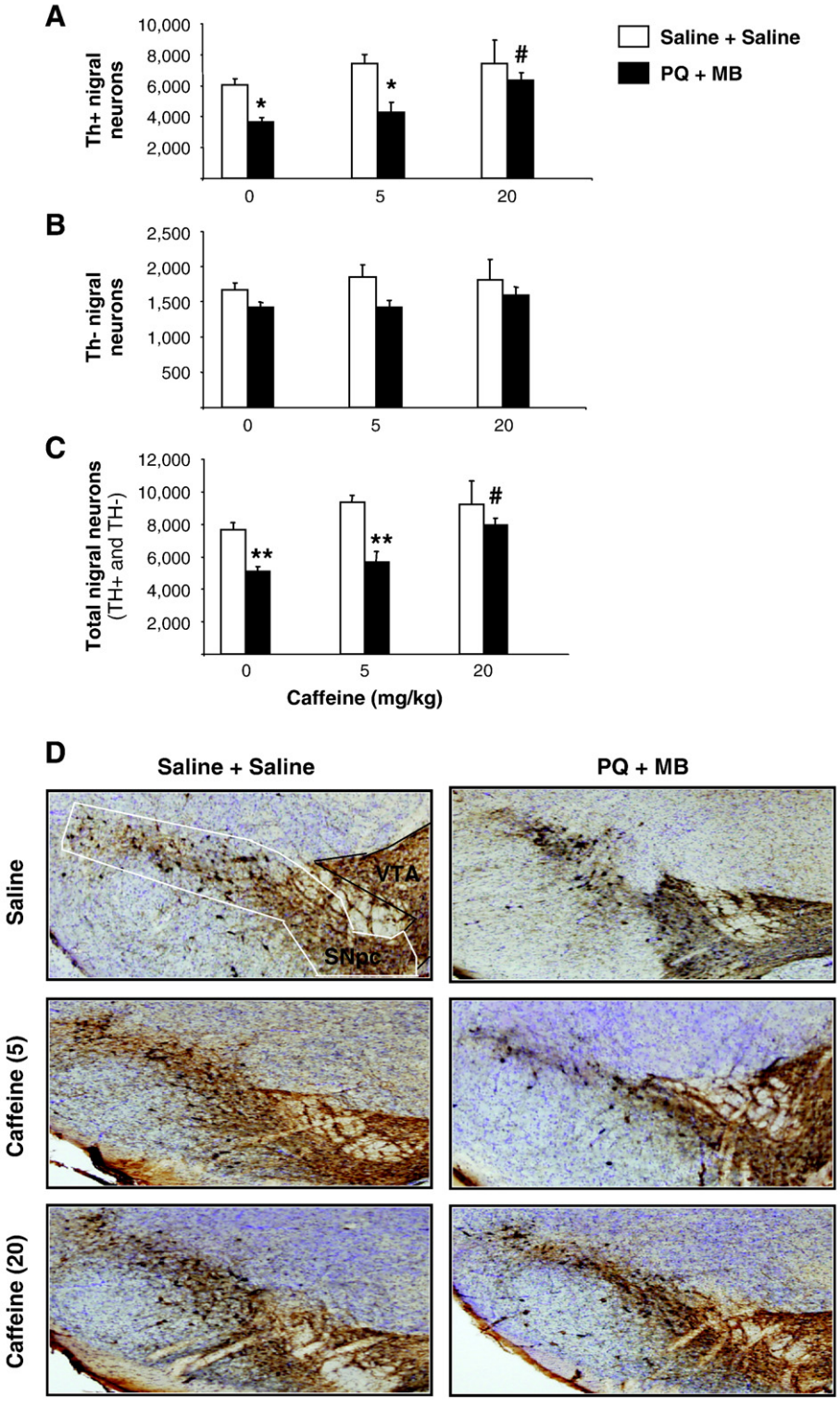


**Fig. 1.** Effect of drug treatments on neuronal cell counts of the substantia nigra pars compacta. Animals were sacrificed 1 week after the last treatment. (A) Total number of TH+ neurons per substantia nigra (bilateral). (B) Total number of TH− neurons per substantia nigra (bilateral). (C) Total neuron counts (TH+ and TH−) per substantia nigra (bilateral). Data are represented as group mean ± SEM. $^{*}P<0.005$, $^{**}P<0.001$ vs. respective control, $^{\#}P<0.05$ vs. saline + paraquat and maneb treatment; one-way ANOVA followed by Bonferroni *post-hoc* analysis. (D) Low-power photomicrographs of representative sections showing PQ and MB-induced loss of TH+ neurons. Low-power (4×) photomicrograph of the substantia nigra pars compacta (SNpc) stained for TH-IR and counterstained with cresyl violet acetate (Nissl) in representative tissue sections from mice pretreated with Saline, Caffeine (5 mg/kg), or Caffeine (20 mg/kg) and treated with Saline + Saline or PQ + MB. Typical delineations for stereological counting are shown for the SNpc and ventral tegmental area (VTA).

All values are expressed as mean ± SEM. Multiple group comparisons were performed using one-way ANOVA followed by Bonferroni *post hoc* analysis.

Combined PQ and MB treatments significantly decreased the number of TH+ neurons in the SNpc by approximately 40% compared to the corresponding saline-treated group (Fig. 1, panels A and D; $P<0.005$). Prior caffeine treatments at 20 mg/kg but not 5 mg/kg provided a significant ($P<0.05$) attenuation in the loss of TH+ neurons, suggesting almost complete neuroprotection (compared to respective caffeine control group) against pesticide-induced neuronal cell loss. Total Nissl-positive neurons (i.e., total number of neurons) showed a similar profile to TH+ neuronal cell counts suggesting that these pesticides targeted the dopaminergic neurons of the SNpc (Fig. 1, panel C). Direct assessment of TH− neurons confirmed that there was no effect of the pesticides on this subpopulation of nigral neurons (Fig. 1, panel B; $P>0.05$). Subanalysis of the data confirmed that changes in total nigral neuron density were comparable to changes in TH+ neuronal cell counts. Assessment of the total volume of the SNpc between groups showed no significant difference.

This study sought to determine whether long-term caffeine pretreatment would afford protection against dopaminergic neuron toxicity in mice chronically exposed to a combined PQ and MB. In this setting, caffeine exposure did in fact reduce the loss of TH+ neurons triggered by pesticide treatment suggesting a neuroprotective effect. However, an alternative explanation is that caffeine upregulated TH as indicated by the tendency toward an elevation of TH+ neurons in caffeine control groups compared to the saline control group. Chronic caffeine treatment at 20 mg/kg has previously been shown to increase expression of TH in nigral neurons (Datta et al., 1996), and recent studies have highlighted the importance of addressing the potential confound of upregulated TH expression in studies of neuroprotection in models of PD (Aumann et al., 2008). If caffeine were indeed upregulating TH expression in our experiment, then the observed increase in TH+ cell counts, albeit not significant, should correlate with a complementary decrease in TH− neurons. Such a conversion of nigral neuron phenotype appeared not to be the case because TH− nigral cells were not reduced in number, indicating that caffeine was neuroprotective in this study. Note that the premature death of two (25%) of the mice in 20 mg/kg caffeine control group represents a potential confound of caffeine-induced systemic toxicity, although it is unlikely to have altered our interpretation.

It is also important to consider the basis of the relatively high mortality (42%) observed after pesticide treatment because interpretation of the results among the surviving mice may not necessarily generalize to those with greater susceptibility to systemic toxicity. Moreover, sublethal systemic toxicity in the surviving mice might have contributed to nigrostriatal toxicity in this model, which would lessen its significance for PD in which there is little evidence of primary systemic pathology. In rats, lethal pulmonary injury is readily observed in response to paraquat exposure (Satomi et al., 2004). In a rat toxicological study that reported substantial mortality after repeated treatment with both paraquat and maneb at the same concentrations used in the present study, half the rats showed progressive weight loss before death or respiratory decline with pulmonary pathology on postmortem analysis (Saint-Pierre et al., 2006). By contrast, the remaining half all regained their normal weight after initial loss and when later sacrificed were found to have normal organ histology, suggesting that lethal organ toxicity in a subset of animals does not necessarily imply substantial toxicity in those that survive. Moreover, in the present study, the fact that mortality after chronic pesticide treatment was identical with and without caffeine pretreatment argues against an interaction between caffeine and pesticide-induced systemic mortality as the basis for the apparent neuroprotection by PD.

Locomotor activity was monitored throughout this experiment to provide a behavioral surrogate of pesticide toxicity. However, PQ and MB treatment failed to reduce basal motor activity after 8 weeks of toxin treatment. Thiruchelvam et al. (2000b) had administered PQ and MB twice a week for 6 weeks to 6-week-old mice and demonstrated a motor deficit 24 hours after the last injection (6 week) time point. This discrepancy between studies may be due to methodological differences. For example, the different substrains of C57Bl/6 mice employed in their study (C57Bl/6J) and ours (C57BL/6NCrl) may contribute to different functional manifestations of neurotoxin-induced injury to nigrostriatal dopaminergic neurons in C57Bl/6 substrains (Heikkila, 1985; Giovanni et al., 1991).

The antiparkinsonian potential of $A_{2A}$ antagonism has been bolstered by convergent epidemiological studies and laboratory data showing that $A_{2A}$ antagonists protect against acute toxin exposure in the MPTP and 6-OHDA models of PD (Xu et al., 2005). Here we demonstrate that the neuroprotective potential of caffeine extends to a chronic and potentially more environmentally relevant 'dual-pesticide' model of PD. These data also extend our preliminary findings in the MPTP model (Oztas et al., 2002) that caffeine can prevent the degeneration *per se* of dopaminergic neurons, as well as their dysfunction.

Although adenosine $A_{2A}$ receptor antagonism likely accounts the protective effects of caffeine in MPTP models of PD (Xu et al., 2005, 2006) the mechanism by which blocking $A_{2A}$ receptors protects dopaminergic neurons remains unsettled. Because $A_{2A}$ receptors are known to facilitate potentially excitotoxic glutamate release in the CNS, it has been suggested that the neuroprotective effects of caffeine and more specific $A_{2A}$ antagonists may be mediated by attenuation of neuronal glutamate release (Xu et al., 2005; Popoli et al., 1995). A key role for neuronal $A_{2A}$ receptors in the chronic neurodegeneration of PD is supported by the recent finding that conditional knockout mice lacking neuronal $A_{2A}$ receptors are resistant to dopaminergic neuron lesions induced by relatively chronic (Carta et al, 2009)) though not acute (Yu et al., 2008) MPTP exposure.

Establishing the ability of caffeine to protect dopaminergic neurons in a chronic pesticide model further supports (but does not prove) the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with the risk of PD. The study also strengthens the rationale for consideration of caffeine and more specific $A_{2A}$ antagonists as therapeutic tools for slowing the underlying degenerative process.

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